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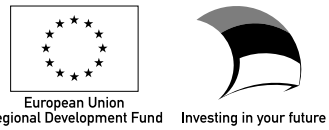

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II. Distinct changes in the behavioural effects of morphine and naloxone in CCK2 receptor-deficient mice. **Rünkorg K,** Veraksitš A, Kurrikoff K, Luuk H, Raud S, Abramov U, Matsui T, Bourin M, Kõks S, Vasar E. Behav Brain Res. 2003 Sept. 144(1–2):125–35

III. Differences in behavioural effects of amphetamine and dopamine-related gene expression in wild-type and homozygous CCK2 receptor deficient mice. **Rünkorg K**, Värv S, Matsui T, Kõks S, Vasar E. Neurosci Lett. 2006 Oct; 406(1–2):17–22

IV. Rimonabant attenuates amphetamine sensitisation in a CCK2 receptor-dependent manner. **Rünkorg K**, Orav L, Kõks S, Matsui T, Volke V, Vasar E. Behav Brain Res. 2012 Jan; 226(1):335–9

Contribution of the author

1. The author performed the behavioural experiments, harvested tissues and performed the radioligand binding studies.
2. The author participated in designing the study, performed the behavioural experiments, harvested tissues, performed radioligand binding studies and participated in writing the manuscript.
3. The author participated in designing the study, performed the behavioural experiments, harvested tissues performed gene expression analysis, performed the statistical analysis and wrote the manuscript.
4. The author participated in designing the study, performed the behavioural experiments, performed the statistical analysis and wrote the manuscript.

ABBREVIATIONS

5-HT_{2A}	serotonin type 2A receptor
ADHD	attention-deficit/hyperactivity disorder
CB1	cannabinoid type 1 receptor
CB2	cannabinoid type 2 receptor
CCK	cholecystokinin
CCK1R	cholecystokinin type 1 receptors
CCK2R	cholecystokinin type 2 receptors
$CCK2R^{-/-}$	CCK_2 receptor deficient homozygous mice line
$CCK2R^{+/-}$	CCK_2 receptor deficient heterozygous mice line
$CCK2R^{+/+}$	wild type mice line
CNS	central nervous system
COMT	catechol-*O*-methyltransferase
Comt	catechol-*O*-methyltransferase gene transcript
Ct	threshold cycle
D_1	dopamine type 1 receptors
D_2	dopamine type 2 receptors
DA	dopamine
DAG	diacylglycerol
DOPAC	dihydroxyphenylacetic acid
Drd1	dopamine type 1 receptor gene transcript
Drd2	dopamine type 2 receptor gene transcript
eCBs	endocannabinoids
ERK1/2	extracellular regulated kinase p44 and p42
GABA	gamma aminobutyric acid
HPLC	high-performance liquid chromatography
Hprt	mouse hypoxanthine–guanine phosphoribosyl transferase gene transcript
HVA	homovanillic acid
IP_3	inositol trisphosphate
Mao	monoamine oxidase A gene transcript
MAPKs	mitogen activated protein kinase
DMCM	methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
OP	opioid peptides
$PI_3\text{-K}$	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol bisphosphate
PKB	protein kinase B
PLC	phospholipase C
POMC	propiomelanocortin
qPCR	quantitative real-time PCR
subNc	substantia nigra pars compacta

subNr	substantia nigra pars reticulate
Tyhy	tyrosine hydroxylase gene transcript
VP	ventral pallidum
VTA	ventral tegmental area

I. INTRODUCTION

Cholecystokinin (CCK) belongs to the largest group of neuropeptides called brain–gut peptides. Originally CCK was discovered by Ivy and Oldberg in 1928 as a hormone from duodenal mucosa mediating gallbladder emptying, and later by Harper and Raper in 1943 as a substance stimulating release of enzymes from the pancreas (Rehfeld and Nielsen 1995). Vanderhaeghen and colleagues (1975) established CCK in the vertebrate brain as a gastrin-like immunoreactivity and it is later considered as the most widely distributed neuropeptide in the mammalian central nervous system (CNS) (Noble et al. 1999). Electrophysiological, biochemical and behavioural studies demonstrated that CCK acts as a neurotransmitter and neurmodulator in the brain. Neuronal CCK is found in numerous brain regions in varying concentrations and it is involved in neurobiology of several physiological and behavioural processes like regulation of feeding, anxiety, pain processing, perception, motivation, learning and memory. CCK serves as a co-transmitter for various mediators in the brain. The interaction of CCK with dopamine (DA) and opioid peptides (OP) has been most widely studied. Hökfelt and colleagues (Hökfelt et al. 1980a) discovered that the subpopulation of mesencephalic dopamine neurons projecting to limbic areas contains CCK. The distribution of both CCK and CCK type 2 receptors (CCK2R) parallels with that of endopioid peptides and opioid receptors in the brain (Gall et al. 1987; Ghilardi et al. 1992; Saito et al. 1980). In different forebrain areas (the amygdala, hippocampus and cerebral cortex) CCK is widely present in gamma aminobutyric acid (GABA) – and glutamatergic neurons containing cannabinoid type 1 receptors (CB1) (Marsicano and Lutz 1999). The physiological relevance of this co-localisation is not clear. The development of the genetically modified mice, lacking CCK2R (Nagata et al. 1996) has been a major step further in order to study the role of CCK in the brain, because the vast majority of effects of CCK in the brain were mediated via CCK2R. The close interaction of CCK with different neuromediator systems in the brain is the reason for the versatile impact of the genetic disruption of CCK2R on the behaviour of mice. The general goal of the present study was to analyze the phenotype of CCK2R deficient mice with particular emphasis on the dopamine, endopioids and endocannabinoids related functions by applying behavioural, pharmacological and molecular biological methods.

2. REVIEW OF LITERATURE

2.1. Cholecystokinin (CCK) in the CNS

Neuronal CCK is present in a variety of biologically active molecular forms (CCK-58, CCK-39, CCK-33, CCK-22, sulphated CCK-8 and CCK-7, un-sulphated CCK-8 and CCK-7, CCK-5, and CCK-4) derived from a three exons consisting, single gene encoded 115-amino-acid pre-pro-CCK precursor molecule. CCK peptides are localised in neuron cell bodies as well as they are concentrated in the nerve endings (Rehfeld and Nielsen 1995). The highest density of CCK neurons is present in the cortical regions and CCK peptides are mainly in nerve processes throughout all the cortical layers. CCK peptides are expressed at high levels in specific subclasses of GABAergic interneurons. CCK's mRNA is expressed in these GABAergic interneurons and also in pyramidal cells (Gallopin et al. 2006). The CCK positive pyramidal neurons are present in the neocortex in laminae II-IV (Toledo-Rodriguez et al. 2005). The hippocampal formation, amygdaloid nuclei, and hypothalamus are rich in CCK neurons. Other brain regions containing moderately developed networks of CCK neurons include the caudate nucleus and putamen, lateral septal nuclei, periaqueductal grey of midbrain and area postrema (Gallopin et al. 2006; Rehfeld and Nielsen 1995; Toledo-Rodriguez et al. 2005). CCK, like the other neuropeptides, is thought to be stored in dense core vesicles, located further away from the active zone and having different release properties and kinetics as compared to classical neurotransmitters (Zhu et al. 1986; Verhage et al. 1991). Depolarisation of nerve terminals can trigger CCK release through Ca^{2+} dependent manner (You et al. 1994). Estrogens may have significant influence on the levels of CCK. CCK positive cells contain clusters of synaptic vesicles which are immunoreactive with estrogen receptor alpha, and estrogen is able to mobilize these vesicles closer to synapses (Hart et al. 2007). CCK levels fluctuate during the various stages of the estrous cycle (Hilke et al. 2007).

Receptors for CCK have been classified by their relative affinity for the natural ligands, their differential distribution in tissues, and their molecular structure (Noble et al. 1999). The main characteristics of the CCK receptors have been listed in Table 1. The CCK type 1 receptors (CCK1R) bind sulphated CCK with a significantly higher affinity than sulphated gastrin or non-sulphated CCK. CCK2R bind gastrin and CCK with almost an equal affinity and discriminate poorly between the sulphated and non-sulphated CCK analogues. In the peripheral tissues CCK1R are presented in the pancreatic acinar cells and D cells of the gastric mucosa, smooth muscle cells of the gallbladder, pyloric sphincter, sphincter of Oddi, gastro-intestinal smooth muscles and enteric neuronal cells, and anterior pituitary corticotrophs (Crawley and Corwin 1994; Wank 1995). Peripheral CCK2R are located in the smooth muscle cells throughout the gastrointestinal tract, parietal, enterochromaffin-like D and chief cells of the gastric mucosa, myenteric plexus neurons, pancreatic acinar cells, monocytes and T lymphocytes (Wank 1995; Wank et al. 1994). The gastrin

receptor mediating acid secretion in the stomach was initially thought to be the third type of CCK receptor. However, so far only two CCK receptor genes have been cloned. The gastrin receptor corresponds to CCK2R located in the gastrointestinal tract and does not constitute the third type of CCK/gastrin receptor.

Table 1. Characterization of the CCK receptor subtypes

Receptor	CCK1R CCK_A/Alimentary/Peripheral	CCK2R CCK_B/Brain/Central
Structure (human)	428 – aminoacid sequence protein (P32238 7TM)	447 – aminoacid sequence protein (P32239 7TM)
Splice variants	No	Long form, short form, Δform
Genetically induced disruption of gene in mice	(Kopin et al. 1999)	(Nagata et al. 1996)
Distribution	Gall bladder, pancreas, pylorus, intestine, spinal cord, vagus nerve, limited brain areas (*nucl. tractus solitarius, area postrema, nucl. interpeduncularis,* posteromedial part of *nucl. accumbens*)	Throughout the brain (with the highest densities in the cerebral cortex, *nucl. caudatus*, anterolateral part of *nucl. accumbens*), vagus nerve, stomach, pancreas
Endogenous ligands according to their affinity of spcific receptor	CCK-8S>>gastrin, CCK-8US>CCK-4	CCK-8S ≥ gastrin, CCK-8US, CCK-4
Agonists	Caerulein (amphibian CCK analogues); A71623; GW5823; JMV-180	Caerulein; CCK-4; Boc-CCK-4; BC197; BC264; CCK-8US; gastrin; RB400
Antagonists	Proglumide; Lorglumide; Devazepide; Lintitript (SR27897); T0632; IQM95333; PD140548	Proglumide, L-365260; L-365260;L-740093; LY288513; CI988; YM022; GV150013; RP73870; LY262691
Intracellular signal transduction	Gq/11; Gs	Gq/11; Gs
Functional effects	In peripheral stimulates: gallbladder contraction, secretion of pancreatic enzymes In peripheral inhibits: gastric emptying, feeding, respiration dopamine–mediated behaviours and dopamine release in shell of nucleus accumbens	In CNS stimulates: neuronal firing rate, nociception, anxiety, respiration In CNS inhibits: dopamine-mediated behaviours and dopamine release in the core of nucleus accumbens

In mammals the genes that encode CCK1R and CCK2R have been organized in a similar manner consisting of five exons and four introns. The receptor genes have homologous exon/intron splice sites. There are three different splice variants for CCK2R. Alternative splicing of exon 4 results in two transcripts that differ by five amino acids within the third intracellular loop. Although the shorter transcript is largely predominant in the stomach, to date, the physiological relevance of these isoforms is unknown (Noble et al. 1999; Song et al. 1993). CCK1R and CCK2R proteins are members of the G protein coupled receptor superfamily and as expected have seven transmembrane spanning domains (Dohlman et al. 1991) The protein sequences of both receptors contain at least three consensus sites for N-linked glucosylation and conserved cysteins in the first and second extracellular loops, which may form a disulfide bridge required for stabilisation of their tertiary structure (Silvente-Poirot et al. 1998). In addition, there is one more cysteine in the C terminus, which may serve as a membrane-anchoring palmitylation site (Noble et al. 1999; O'Dowd et al. 1988).

The intracellular signal-transduction cascade of the CCK1R in pancreatic acinar cells is well known but it is rather poorly characterized for CCK2R, mainly because of the difficulty in working with isolated neurons or isolated gastric mucosa cells. Nevertheless, it has been shown that both CCK1R and CCK2R are coupled to a pertussis toxin-insensitive Gq protein family, which includes α_q, α_{11}, and α_{14} proteins and thereby they are able to activate phospholipase C (PLC). PLC cleaves phosphatidylinositol bisphosphate (PIP_2) to form inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to the intracellular ligand-gated Ca^{2+}channels and thereby initiates the release of sequestered Ca^{2+}. DAG activates protein-kinase C. The effect of CCK on neuronal excitability of pyramidal neurons in the enthorhinal cortex was studied, and the results showed that CCK increased firing frequency of neurons. This effect is mediated via CCK2R, involving G-proteins, PLC; and transient receptor potential channel 5 (Wang et al. 2011). CCK1R are coupled to the phospholipase A_2/arachidonic acid pathways (Wu et al. 1997). It is likely that CCK1R are coupled with both G_s and G_q protein, because activation of CCK1R can also lead to an increase in the adenylyl cyclase signal transduction cascade (Marino et al. 1993; Yule et al. 1993). CCK might via CCK1R stimulate Ras, mitogen activated protein kinase (MAPKs) and c-Jun NH_2-terminal kinase (JNKs) and due to that activate cell proliferation (Dabrowski et al. 1996; Tateishi et al. 1998). There is some evidence that CCK2R are also coupled to the MAPKs (Taniguchi et al. 1994), and to the arachidonic acid, activating pertussis toxin insensitive or sensitive G protein/phospholipase pathways (Pommier et al. 1999; Williams 2001). In the pancreatic cells CCK activates extracellular regulated kinase p44 and p42 (ERK1/2) by binding to CCK1R or CCK2R and it involves as well PKC-mediated activation of multiple forms of Raf (Seva et al. 1997; Williams 2001). CCK2R activation in the pancreatic cell line promotes cell survival through the induction of phosphoinositide 3-kinase and protein kinase B (PI_3-K/PKB) signalling pathways (Todisco et al. 2001). In

the rat brain neuroblasts CCK induces, probably through CCK2R, activation of the intracellular signal cascade leading to tyrosine phosphorylation of the proteins and stimulation both the ERK1/2 and the PI_3-K/PKB intracellular pathways involved in the cell survival and proliferation (Langmesser et al. 2007).

2.2. Interaction of CCK with dopamine system

Dopamine is a catecholamine synthesized from the essential amino acid tyrosine in common biosynthetic pathway with noradrenaline and adrenaline. It is a predominant catecholamine neurotransmitter in the mammalian central nervous system where it controls a variety of functions including cognition, emotion, locomotor activity, hunger, satiety and endocrine functions. The action of dopamine is mediated via five distinct (D_1, D_2, D_3, D_4 and D_5) G protein-coupled receptor subtypes. Two D_1-like receptor subtypes (D_1 and D_5), coupled to the G-protein G_s, activate adenylyl cyclase and stimulate cAMP formation. Other receptor subtypes (D_2, D_3 and D_4) belong to the D_2-like receptor subfamily which inhibit adenylyl cyclase and activate K^+ channels (Missale et al. 1998).

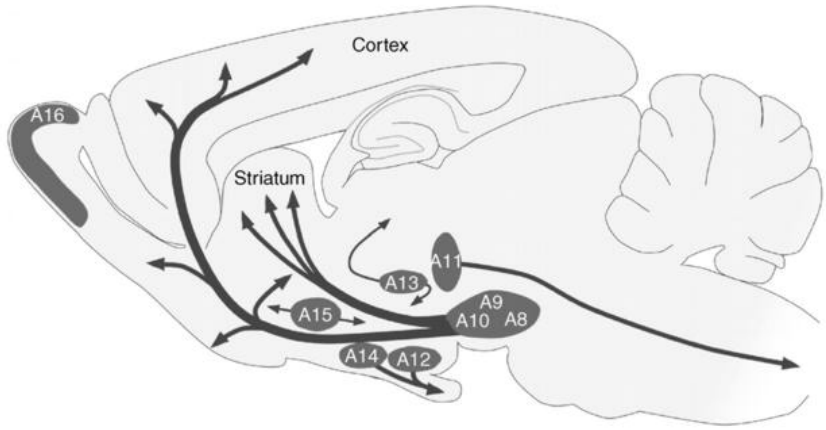

Figure 1. Distribution of DA neuron cell groups in the rodent brain in a schematical sagittal view. The principal projections of the DA cell groups are illustrated by arrows. A modified illustration from Bjorklund and Dunett (Bjorklund and Dunnett 2007).

The mammal's brain contains nine distinctive areas of dopamine neurons distributed from the midbrain to the olfactory bulb (Figure 1.). The numbering of these cell groups, from A8 to A16, was introduced in the classic study of Dahlström and Fuxe in 1964, and it is still valid (Dahlstrom and Fuxe 1964). In the midbrain the lateral A9 subgroup corresponds to neurons of the substantia nigra pars compacta (subNc), which have prominent projections to the dorsal striatum (the nigrostriatal dopaminergic pathway) and are involved in the

control of voluntary movement. The medially located A10 and A8 subgroups form the ventral tegmental area (VTA) and retrorubral field, respectively. Neurons in these subgroups prominently innervate the ventromedial striatum and prefrontal cortex, as part of the mesolimbic system, and are involved in the regulation of emotions and reward (Bjorklund and Dunnett 2007; Van den Heuvel and Pasterkamp 2008). Among the diencephalic and hypothalamic (A11 to A15) areas the A12 group is located in the arcuate nucleus of the hypothalamic median eminence and is involved in inhibitory control of the release of prolactin from the lactotroph cell in anterior lobe of the pituitary, but dopamine D_2 receptors are also found in pituitary somatotroph, thyreotroph as well as in gonadotroph cells (Pivonello et al. 2007). Dopaminergic periglomerular cells in the olfactory bulb belong to the area A16 and interplexiform cells in retina to the area A17 (Marin et al. 2005).

CCK is co-localized with dopamine in the most of dopaminergic neurons of the VTA projecting to the mesolimbic structures (Hökfelt, Rehfeld et al. 1980). The main CCKergic input of the nucleus accumbens (NAc) originates from the ventral midbrain. The accumbal rostral pole is equally innervated by CCK neurons projecting from both subNc and the ventral VTA, whereas the primary source of CCK innervation of the accumbal core is the VTA. Most of the CCKergic neurons projecting to any part of the accumbal compartments contain also DA (Lanca et al. 1998). CCK is shown to modulate dopaminergic neurotransmission in the mesolimbic structures. This interaction is complex and depends on the route of administration of CCK agonists and the subtype of CCK receptor involved (Altar and Boyar 1989; Crawley and Corwin 1994; Ladurelle et al. 1997). CCK1R are mostly localized in the caudal shell region of the NAc , whereas CCK2R are mostly localized in the rostral core in the NAc (Marshall et al. 1991). Pharmacological and behavioural studies demonstrate that CCK1R and CCK2R mediate the opposite effects of CCK on the activity of DA neurons in mice and rats. In general, in the caudal shell area of the NAc, CCK1R stimulation induces agonistic-like action on the effects of DA, such as increased firing of DA neurons (Carr and White 1986), potentiated K^+-stimulated DA release (Vickroy et al. 1988) and increased DA turnover (Kariya et al. 1994). Conversely, stimulation CCK2R in the rostral core area of the NAc has DA antagonistic-like effects, such as attenuated K^+-stimulated DA release, decreased extracellular DA concentrations (Voigt et al. 1985) and turnover (Dahlstrom and Fuxe 1964; Voigt et al. 1985). CCK-8S reduces via CCK2R the apparent affinity of D_2 agonists in the nucleus accumbens and caudate putamen (Li et al. 1995). Altar and colleagues showed that CCK2R activation suppresses both basal and augmented DA release. The increase in DA release after amphetamine administration is attenuated by the central administration of BOC-CCK-4 or CCK-8US (the agonists of CCK2R) and by the central or systemic treatment with CCK-8S (Altar and Boyar 1989). On the other hand Ferrero and colleagues demonstrated that CCK-8S could via activation of the CCK2R receptor inhibit function of the pre-synaptic D_2 receptors in nucleus accumbens and thereby reduce local DA release (Ferraro et al. 1996). CCK2R stimulation

opposes functionally as well the post-synaptic effects of DA in the nucleus accumbens (Weiss et al. 1989; Yim and Mogenson 1991). An additional indirect effect on dopaminergic functions of CCK is mediated via influence on the endopioid system.

2.3. Opioid peptides in the CNS, interaction of CCK and dopamine with the endopioid system

Opioid peptides (OP) are endogenous ligands for the opioid receptors (Evans 2004). Pharmacological studies have defined three main opioid receptor subtypes, respectively mu, delta and kappa receptors. More than 20 different identified opioid peptides (Table 2) possess a diverse affinity and specifity for these three receptor types. All mammalian opioid peptides have an N-terminal enkephalin sequence (Tyr-Gly-Gly-Phe-Met/Leu), and are derived mainly from three opioid protein precursors pro-opiomelanocortin (POMC), proenkephalin, prodynorphin) by selective proteolytic cleavages predominantly at basic and paired basic residues. Each opioid peptide precursor has a unique pattern of expression, with pro-opio-melanocortin transcripts restricted mainly to the pituitary, the arcuate nucleus of the hypothalamus and some cells in the nucleus of the solitary tract, whereas both pro-enkephalin and prod-dynorphin have a considerably more widespread distribution and are located in the brain regions that regulate the extrapyramidal motor function, the cardiovascular and water balance system, eating, sensory processing, and pain perception (Peckys and Landwehrmeyer 1999).

Opioid receptors are highly homologous to each other, have seven transmembrane-spanning domains and belong to the family of the G-protein coupled receptors (Zaki et al. 1996). There are suggestions that more than three opioid receptor genes could exist. However, despite that no other opioid receptor genes were identified by molecular cloning besides the opioid like receptor (ORL_1 or NOP_1) and this was not considered a classical opioid receptor since it did not bind the classical opioid receptor ligands such as naloxone. Endogenous ligand of these receptors, orphanin FQ is derived from a protein precursor different from other opioid peptides. Some other mechanisms, like RNA editing or different splicing, could create receptor diversity from single genes. Alternative splicing at the C-terminus has been observed in rodent mu-receptor and this could provide heterogeneity in opioid receptor molecules and could modify receptor functioning, because this region is important in transduction of the signal following receptor activation (Pasternak 2004).

Table 2. Endogenous opioid peptides.

Endogenous opioid peptides				
Receptor	Precursor	Endogenous opioid	Amino Acid Sequence	Source
μ (MOR, OP3) mu-receptor	Pro-opiomelanocortin	β-endorfiin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu	Mammalian brain
	β-Casein (bovine)	β-Casomorphin-5	Tyr-Pro-Phe-Pro-Gly	Bovine milk
		β-Casomorphin-7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	
		Morphiceptin	Tyr-Pro-Phe-Pro-NH2	
	β-Casein (human)	β-Casomorphin-5	Tyr-Pro-Phe-Val-Glu	Human milk
		β-Casomorphin-7	Tyr-Pro-Phe-Val-Glu-Pro-Ile	
	Hemoglobin	Hemomorphin-4	Tyr-Pro-Trp-Thr	Human blood
		Hemomorphin -7	Tyr-Pro-Trp-Thr-Gln-Arg-Phe	
	Unknown	Endomorphin-1	Tyr-Pro-Trp-Phe-NH2	Bovine brain, human brain cortex
		Endomorphin-2	Tyr-Pro-Phe-Phe-NH2	
	Unknown	Tyr-MIF-1	Tyr-Pro-Leu-Gly-NH2	Bovine brain, human brain cortex
		Tyr-W-MIF-1	Tyr-Pro-Trp-Gly-NH2	
δ (DOR, OP1) delta-receptor	Proenkephalin	Met-enkephalin	Tyr-Gly-Gly-Phe-Met	Mammalian brain
		Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	
			Tyr-Gly-Gly-Phe-Met-Arg-Phe	
			Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu	
K (KOR, OP2) kappa-receptor	Prodynorphin	Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln	Mammalian brain
		Dynorphin A(1–8)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile	
		Dynorphin B	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr	
ORL1 (NOP1)	Pronociceptin	Nociceptin/orphanin FQ	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln	Mammalian brain

Opioidergic, dopaminergic and CCK-ergic systems are neuroanatomically and functionally closely related. Phasic dopamine release has been shown to increase endopioid levels (Roth-Deri et al. 2003), whereas tonic dopamine decreases endopioid levels (King et al. 2001; Zubieta et al. 2003) in NAc. Conversely, OP potentiate phasic DA release in the striatum by inhibiting local GABAergic interneurons in the VTA (Johnson and North 1992; Nugent et al. 2007). The CCK and endopioid systems are in the antagonistic interaction if the regulation of behaviour is concerned. The administration of CCK attenuates, whereas the selective CCK2R antagonists enhance, morphine-induced anti-nociception in rodents (Faris et al. 1983; Lavigne et al. 1992; Noble et al. 1995). CCK2R antagonists, but not CCK1R antagonists, facilitate the antidepressant-like effect induced by opioid peptides in the conditioned suppression of motor activity in mice (Smadja et al. 1995). Moreover, the selective CCK2R antagonists potentiate the rewarding effect of morphine in the place conditioning paradigm (Higgins et al. 1992; Valverde et al. 1996). The CCK2R antagonists L-365,260 and PD-134,308 are shown to attenuate the place aversion induced by naloxone in morphine-dependent rats, whereas the CCK1R receptor antagonist devazepide is ineffective in this respect (Valverde and Roques 1998). A morphine-induced increase in the locomotor activity can also be antagonised by the pre-treatment with CCK (Schnur et al. 1991). These findings suggest that CCK may act, via CCK2R, as an endogenous anti-opioid peptide. It is also noteworthy that the distribution of both CCK and CCK2R parallels with that of OP and opioid receptors in the brain (Gall *et al.* 1987; Ghilardi *et al.* 1992; Saito *et al.* 1980).

2.4. Endocannabinoids in CNS and interaction of CCK with endocannabinoid system

The eCBs (anandamide and 2-arachidonylglycerol), endogenous ligands of the cannabinoid type 1 receptor (CB1) and cannabinoid type 2 receptor (CB2) receptors (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995), are produced from phospholipid precursors "on demand", in a Ca^{2+}-dependent manner when the neurons are stimulated with membrane-depolarizing agents (Di Marzo et al. 1994). The release of eCBs immediately follows their biosynthesis. The neuronal eCBs are inactivated by a rapid elimination process consisting of selective uptake into the cell and subsequent degradation by fatty acid amide hydrolase or monoacylglycerol lipase (van der Stelt and Di Marzo 2003). CB1 receptors are abundant G-protein goupled receptors in the CNS. Activation of presynaptic CB1 receptors results in the inhibition of both excitatory and inhibitory neurotransmitter release. CB1 receptors can be located on several neurones, including GABA-, glutamate-, serotonin- and dopaminergic neurons (Freund et al. 2003; Szabo and Schlicker 2005; Pacher et al. 2006; Lazary et al. 2011; Umathe et al. 2011). CB1 receptors are also expressed in the periphery and are present in several non-neuronal tissues (e.g.

the gastrointestinal system, reproductive system, cardiovascular system, adipocytes, liver, skeletal muscle cells and pancreas). These receptors are directly involved in the orexigenic effect of eCBs as well as in the regulation of metabolism, body weight as well as insulin resistance and are therefore the main targets in the development of new drugs for obesity and metabolic syndrome treatment (Cota et al. 2003). Rimonabant (SR141716A, Acomplia, Sanofi-Aventis), the first selective CB1 antagonist or inverse antagonist (Rinaldi-Carmona et al. 1994), was developed as an anti-obesity agent on the premise that blocking central eCB activity might reduce food intake (Pacher et al. 2006). The potent anti-obesity efficacy of rimonabant was demonstraeted by several clinical studies (Despres et al. 2009; Hampp et al. 2008), and dyslipidaemias, diabetes and metabolic syndrome were also ameliorated (Rosenstock et al. 2008). Rimonabant was not only proposed for the treatment of obesity, metabolic conditions as well as addictive disorders (smoking cessation) (Cahill and Ussher 2007; Rigotti et al. 2009). However, it induced significant psychiatric side effects, namely anxiety and depression, and therefore it was withdrawn from the European market in October 2008 (Kirilly et al. 2012).

Cannabinoid type 2 receptors (CB2) are expressed in the immune system and in the brain microglia during neuro-inflammation. CB2 receptor was isolated by a PCR-based strategy designed to isolate GPCRs in differentiated myeloid cells. The CB2 has 44% amino acid identity with CB1, and a distinct yet similar binding profile, and thus represents a distinct receptor subtype (Munro et al. 1993). CB1 and CB2 receptors belong to the seven-transmembrane G-protein coupled receptor family. Their activation typically leads to the inhibition of adenylate cyclase, consequently closing calcium channels, opening potassium channels and stimulating protein kinase A (Freund et al. 2003).

Like CCK eCBs take part in the modulation of feeding behaviour, behavioural expression of anxiety, pain, learning and memory (Noble *et al.* 1999; van der Stelt and Di Marzo 2003). In various forebrain areas (the amygdala, hippocampus and cerebral cortex) CCK is widely present in GABA- and glutamatergic neurons containing CB1 receptors (Marsicano and Lutz 1999). The functional interaction between CCK and eCBs seems to be in most cases antagonistic. eCBs can function as retrograde messengers in the synaptic level (Chhatwal et al. 2009), reducing via CB1 receptors the excitability of presynaptic neurons. Indeed, the activation of CB1 receptors has been shown to inhibit potassium-evoked CCK release in the hippocampus (Beinfeld and Connolly 2001).

2.5. Phenotype of CCK2 receptor deficient mice

Our study was performed on gene targeted CCK2R deficient mice line ($CCK2R^{-/-}$), with original background of 129Sv/C57Ll/6 and which were originally generated by replacing a part of exon 2 and exons 3, 4 and 5 (Nagata et al. 1996). The adult $CCK2R^{-/-}$ mice are fertile, have a normal body weight

and are without obvious abnormalities in their general appearance in comparison with their wild-type littermates ($CCK2R^{+/+}$). CCK2R mRNA is absent in total RNA samples extracted from the $CCK2R^{-/-}$ mice cerebral cortex, brain basal ganglia and stomach, which abundantly express this transcript in the $CCK2R^{+/+}$ mice examples. The lack of a functional CCK2R protein in whole brain and pancreas preparations was proved by ^{125}I-CCK-8 binding assay. CCK2R/gastrin receptors on the gastric parietal cells have an important role on the regulation of gastric acid secretion. A marked atrophy in the $CCK2R^{-/-}$ samples became evident in the macroscopic and histological studies of the gastric mucosa. The number of parietal cells was decreased in the fundic glands and the expression of H^+,K^+-ATPase mRNA was significantly reduced in the parietal cells. The basal gastric acid output in the $CCK2R^{-/-}$ mice was remarkably inhibited compared with that of the $CCK2R^{+/+}$ mice and as expected, serum gastrin levels in the adult $CCK2R^{-/-}$ mice, fasted for 6 hr, were about 5 times higher than those in the wild-type mice (Nagata et al. 1996). The gastric emptying with a non-nutrient liquid load was investigated and it was significantly enhanced in the $CCK2R^{-/-}$ mice as compared with wild type littermates (Miyasaka et al. 2004).

The effect of CCK2R deficiency in memory process was studied in a Y maze. During the two-trial recognition memory task after a 2 hr inter-trial interval, wild-type mice spent significantly more time in the novel arm than mutant mice, indicating a decreased performance of mutant mice group (Sebret et al. 1999).

The CCK2R deficient mice showed an increased spontaneous locomotor activity and the administration of the D_2 selective antagonist sulpiride completely abolished this effect (Dauge et al. 2001). By the Western blot analysis the dopamine D_2 receptor expression in the nucleus accumbens was studied and it was significantly lower in the $CCK2R^{-/-}$ than in wild type male mice (Miyasaka et al. 2005).

The existence of physiologically relevant interactions gives a reason to analyse the effect of CCK2R gene invalidation on the endopioid system. The absence of negative feedback control in genetically modified mice, normally performed by CCK2R stimulation, results in an up regulation of the endopioid system with a positive coupling of the μ and δ opioid receptors to the adenylyl cyclase pathway. It was evidenced with an increased locomotor response to the administration of morphine or the enkephalin-degrading enzyme inhibitor RB 101. Moreover, much more severe withdrawal syndrome was observed in these genetically modified animals after chronic morphine treatment. These mice showed spontaneous hyperalgesia and hyposensitivity toward the antinociceptive effects of morphine and RB101 in the hot-plate jump latency test (Pommier et al. 2002). A reduced pain sensitivity of CCK2R deficient mice in the plantar analgesia and hotplate tests was established by Veraksitš and colleagues (Veraksitš et al. 2003). However, in this study CCK2R deficient mice seemed to have a reduced tolerance to painful stimuli as measured by the jump latency in the hotplate test. In the plantar analgesia test the antinociceptive

action of morphine is significantly stronger in mutant mice, whereas in the hotplate test the effect of morphine remained unchanged compared with wild-type littermates. The repetitive administration of morphine results in an easier development of motor sensitization in the $CCK2R^{-/-}$ group (Dauge et al. 2001).

CCK is implicated in the regulation of anxiety and anxiogenic-like action of CCK agonists is mediated via CCK2R (Noble et al. 1999). The activity of GABA-ergic system is increased in the brain of mice lacking CCK2R. Female $CCK2R^{-/-}$ mice displayed an increased exploratory activity in the dark-light exploration test (Raud et al. 2005) and elevated plus-maze (Areda et al. 2006; Raud et al. 2003). The wild-type littermates displayed higher sensitivity to the anxiolytic-like action of diazepam compared to the homozygous $CCK2R^{-/-}$ animals (Raud et al. 2005; Raud et al. 2003). The administration of methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM) an inverse agonist of benzodiazepine receptors, caused opposite changes in the exploratory behaviour of the wild type and $CCK2R^{-/-}$ female mice in the dark-light exploration test. An increase in exploratory activity was established in the female wild-type animals, by contrast, the administration of DMCM caused a dose-dependent reduction of exploratory behaviour in the $CCK2R^{-/-}$ female mice (Raud et al. 2005). The anxiogenic-like effect of cat odour was studied and it was found that the exposure to cat odour caused a significant anxiogenic-like action in the CCK2 receptor deficient female mice. This effect was not seen in the wild-type female animals, displaying a significantly lower exploratory activity in the plus-maze (Areda et al. 2006). The higher exploratory activity of the CCK2R deficient female mice was accompanied by an increased expression of mu-opioid receptor and CCK1R mRNA in the frontal cortex and midbrain. The CCK gene expression was reduced in the frontal cortex and mesolimbic area of female mice, lacking CCK2R (Areda et al. 2006). In the $CCK2R^{-/-}$ female mice group, after the exposure of cat odour, some significant alterations in the gene expressions were established. The increase of POMC gene was remarkable in the temporal lobe, mesencephalon and mesolimbic area. Cat odour exposure elevated the expression of the melanocortin type 3 receptor gene in the temporal lobe and frontal cortex of homozygous mutant female mice. In the temporal lobe cat odour also increased the expression of delta opioid receptors and pro-enkephalin genes in homozygous female mice (Areda et al. 2006). It has been shown that the $CCK2R^{-/-}$ mice do not develop eCB-sensitive stress induced analgesia, because the analgesic response is antagonized by opioid antagonist naloxone, but not by CB1 receptor antagonist rimonabant. The stress, induced by inescapable electric foot-shocks, caused up-regulation of eCB-related genes in the lumbar spinal cord and mesolimbic area of wild-type mice, whereas no change occurred in the $CCK2R^{-/-}$ animals (Kurrikoff et al. 2008).

3. AIMS OF THE STUDY

Based on the literature review it is clear that CCK closely interacts with various neuromediator systems in the brain and, therefore, the genetic invalidation of CCK2R could have versatile impact on the behaviour of mice. In order to extend our pool of knowledge about the role of CCK in the regulation of behaviour the following specific tasks were put forward:

1. To study the impact of CCKergic mechanisms on functional, neurochemical and molecular characteristics of brain dopaminergic neurons.
2. To describe whether invalidation of CCK2R in mice changes the activity of endopioid system and adaptive behaviour in mice.
3. To determine the functional role of eCB-s in the development of amphetamine induced sensitization and possible involvement of CCK2R.

4. MATERIALS AND METHODS

4.1. Animals

The CCK2R deficient mice with original background 129 Sv/C57Bl/6 were generated by replacing a part of exon 2 and exons 3, 4 and 5 (Nagata et al. 1996). Breeding and genotype analysis were performed at the Department of Physiology of the University of Tartu. Genotyping was carried out by means of polymerase chain reaction (PCR) using two pairs of primers – HE2F (TGG AGT TGA CCA TTC GAA TCA C) and LacZrev (GTG CTG CAA GGC GAT TAA GTT G) were designed to detect the mutant allele, and HE3F (TAT CAG TGA GTG TGT CCA CTC T) and HE3R (ACA TTT GTT GGA CAC GTT CAC) were designed for the wild-type allele. The following protocol was used for PCR: 96^{o}C for 10 min (initial denaturation); 96^{o}C for 50 s, 60^{o}C for 50 s, and 72^{o}C for 2 min (25 cycles); and 72^{o}C for 10 min (final amplification). PCR products were stored at 4^{o}C until electrophoresis. Mutant mice were crossed back three times (paper I) six times (paper II), ten times (paper III) and twelve times (paper IV) to the C57Bl/6 background to minimize any possible genetic effects from the 129sv strain. Altogether 377 male homozygous CCK2R$^{-/-}$, 98 male heterozygous CCK2R$^{+/-}$ and 381 male wild-type CCK2R$^{+/+}$ adult (3–5 months old) mice were used in the behavioural and radioligand binding studies. The heterozygous CCK2R$^{+/-}$ mice were used only in the first study. In the following studies we reduced the number of animals used in experiments and compared only the homozygous CCK2R$^{-/-}$ and the wild-type CCK2R$^{+/+}$ mice. The mice were kept in the animal house at 20±2^{o}C under a 12-h/12-h light/dark cycle (lights on at 0700). Tap water and food pellets were available ad libitum. All animal procedures were approved by the Animal Care Committee of the University of Tartu in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

4.2. Drugs

All drug solutions were administered intraperitoneally in volume 10 ml/kg. Amphetamine sulphate (Sigma) (Papers I, III and IV), apomorphine hydrocloride (Sigma) (Paper I), morphine sulphate (Boehringer-Ingelheim) (Paper II), and naloxone hydrochloride (Sigma) (Paper II), were dissolved in sterile, pyrogen free, 0,9% solution of sodium chloride in water (saline). Rimonabant (Sanofi–Aventis) was dissolved in vehicle (saline, 5% dimethyl sulphoxide, and a few drops of Tween-80) (Paper IV).

4.3. Analysis of dopamine and its metabolites in the mouse brain samples (Paper I)

Mice were decapitated; the brains were rapidly removed and cooled in liquid nitrogen. The following brain areas were dissected from the sections according to the Franklin and Paxinos Mouse Brain Stereotaxic Atlas (Paxinos 1997): the cerebral cortex, both striata, and the hypothalamus. The dissected tissues were frozen in liquid nitrogen and stored at –80°C until sample preparation. The brain samples were homogenised in 10–50 volumes of 0.1 N $HClO_4$, depending on tissues. The homogenates were centrifuged at 16,500 rpm (15 min +4°C, Heraeus Sepatech, Biofuge 17RS). The supernatant was removed, separated into two individual Eppendorf tubes and stored at –80°C until analysed. Two different high-performance liquid chromatography (HPLC) runs determined DA and their metabolites: one assay for DA and its metabolite dihydroxyphenylacetic acid (DOPAC), and another assay for measuring homovanillic acid (HVA). Both chromatographic systems consisted of an isocratic Waters 510 pump (Waters), a Waters 717 plus auto-sampler with cooler (Waters), a reverse-phase C18 column (Ultrasphere ODS, 4.6×250 mm, 5 µm particle size, Beckmann) and Hewlett Packard HP-3396A integrators. In the HPLC system 1, the detector (ESA Coulochem II, ESA) was equipped with a 5021 (ESA) conditioning cell and a two-channel (Det 1 and Det 2) 5011 (ESA) analytical cell. The potentials applied were +0.10 V (conditioning cell), +0.375 V (Det 1) and –0.350 V (Det 2). The sensitivities were set at 100 nA (Det 1) and 5 nA (Det 2). In the HPLC system 2, the detector (ESA Coulochem 5100A, ESA) was equipped with a 5021 conditioning cell (+0.10 V) and a 5014A analytical cell (+0.35 V, Det 1). The sensitivity was set at 15×100. The detection limits were 18 fmol for DA and DOPAC in HPLC 1 and 100 fmol for HVA in HPLC 2. The mobile phase consisted of a sodium acetate buffer (0.1 M), citric acid (0.1 M), methanol (7.5%) and sodium octyl sulphate 45 mg/l for the first HPLC method and 90 mg/l for the second. The pH of the mobile phase was adjusted to 3.6 with phosphoric acid, and the flow rate was 1.1 ml/min.

4.4. Radioligand binding studies (Papers I and II)

The mice used in the radioligand binding study were not exposed to the behavioural testing. After the decapitation of mice their brains were quickly dissected on ice. The striatum and mesolimbic structures were used for studying the parameters of dopamine D_2 receptors, whereas the cerebral cortex was used for serotonin 5-HT_2 receptor studies (paper I). The opioid receptors were analysed in the cerebral cortex (including the frontal and parietal cortices), the striatum, mesolimbic (*nucleus acumbens* and *tuberculum olfactorium*) and the hippocampus (paper II). The dissected tissues were frozen and stored at –80°C until sample preparation. The radioligand binding studies were performed in accordance with the method described by Kõks and colleagues (Kõks et al.

1997). Each experiment was repeated at least three times. The brain structures from six mice were pooled. The brain tissue was homogenized in 20 volumes of ice-cold 50 mM Tris-HCl (pH 7.4 at 4° C) using a Potter-S glass-teflon homogenizer (1000 rpm, 12 passes). The membranes were washed twice in the same buffer by centrifugation (48000 × g for 20 min) and re-suspension. After the last centrifugation crude brain membranes were suspended in an appropriate incubation buffer at the concentration of 10 mg wet weight/ml. After the incubation time the binding reaction was determined by rapid filtration over Whatman GF/B filters, thereafter washed with 9 ml ice-cold buffer. The filters were dried and left overnight in a Wallace High Safe III scintillation cocktail. Radioactivity was assayed by liquid scintillation spectrometry (Wallac β-scintillation counter) at 50 per cent efficiency level. The protein content was measured according to the Bradford method (Bradford 1976). The saturation curves of radioligand binding were analysed by using GraphPad Prism (Version 3.00) for Windows software.

4.4.1. [^{3}H]-spiperone binding for serotonin (5-HT$_{2A}$) and dopamine (D$_2$) receptors (Paper I)

After final washing the crude brain membranes were homogenised in the incubation buffer consisting of 50 mM Tris; 120 mM NaCl; 5mM KCl; 2 mM $CaCl_2$; 1 mM MgCl; pH 7,4 adjusted with HCl. The parameters of dopamine and serotonin receptors were determined in the presence of 0.06–2 nM [^{3}H]-spiperone (specific activity 107 Ci/mmole) (Amersham Radiochemicals) incubated at 37°C for 30 min. Raclopride (1 μM) (Astra), an antagonist of dopamine D_2, was added to determine the non-specific binding of dopamine D_2. Ritanserin (1μM) (RBI), an antagonist of 5-HT_2, was used to detect the non-specific binding of 5-HT_2.

4.4.2. [^{3}H]-diprenorphine binding for opioid receptors (Paper II)

After the last centrifugation in the washing step the crude brain membranes were resuspended in the incubation buffer (50 mM Tris; 120 mM NaCl; 5mM KCl; 2 mM $CaCl_2$; 1 mM MgCl; pH 7.4 adjusted with HCl). The parameters of the opioid receptors were determined in the presence of 0.05–2.5 nM [^{3}H]-diprenorphine (specific activity 58.0 Ci/mmole) (Amersham Radiochemicals) at 23°C for 60 min. Naloxone hydrochloride (1 μM) (RBI), an antagonist of opioid receptors, was added to determine the non-specific binding of opioid receptors.

4.5. Gene expression analysis (Paper III)

In gene expression studies, quantitative real-time PCR (qPCR) was applied. The animals used for the behavioural sensitization experiment were exploited in this study. Forty-eight hours after the last amphetamine or saline injection mice were killed with cervical dislocation. Three brain areas (the striatum, the mesolimbic area and the mesencephalon) were quickly dissected on ice. For the dissection of the striatum and the mesolimbic area the first transverse section was made at the level of the *chiasma opticum*. The second transverse section was performed 2 mm anterior from the line crossing the *chiasma opticum*. The dissection of the striatum and the mesolimbic area was performed according to the coordinates of the Franklin and Paxinos Mouse Brain Stereotaxic Atlas (Paxinos 1997). The mesolimbic area includes the NAc, VP, *tuberculum olfactorium*, and the islands of Calleja. For the dissection of the mesencephalon, the cerebellum was removed and the first transverse section was made at the level of the posterior border of the *corpora quadrigemina* (*colliculus inferior*). The second transverse section was made behind the posterior border of the hypothalamus. The mesencephalon includes a number of various structures (the *corpora quadrigemina*, periaqueductal gray, oculomotor nuclei, *etc.*), but for the purposes of our study two nuclei containing dopaminergic neurons (*substantia nigra* and the VTA) are especially important. The dissected tissues were frozen in liquid nitrogen and stored at −80 C until sample preparation. Total RNA was extracted using TRIzol-Reagent (Invitrogen) according to the manufacturer's protocol. Total RNA from different animals of each group was pooled. Pooling was performed to minimize the fluctuations resulting from individual differences. Every experimental group (n=13) was pooled as follows: three pools including samples from three mice and one pool including samples from four animals. Consequently, the number of independent samples in each case was four. Forty-eight different pools of total RNA (three brain structures, four groups of animals and four samples) were received. As a rule, experiments with wild-type and homozygous mice were conducted in parallel. The first strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). The expression levels of the following gene transcripts were measured: catechol-*O*-methyltransferase (*Comt*), monoamine oxidase A (*Mao*), tyrosine hydroxylase (*Tyhy*), dopamine D_1 receptor (*Drd1*) and dopamine D_2 receptor (*Drd2*) (primers have been presented in Table 3.). The qPCR was performed using ABI Prism 7900 Sequence Detection System (PE Applied Biosystems) equipment and ABI Prism 7900 SDS Software. We used SYBR green I qPCR™ Core Kit (Eurogentec) for all reactions. PCR was set up by the following steps: 95 C for 10 min; 95 C 15 s and 60 C for 1 min, repeated for 40 cycles. Melting curve analysis of the amplification product was performed at the end of each reaction to confirm the quality of the PCR. All reactions were performed in the final volume of 10 µl, using 50–100 ng of cDNA. Mouse hypoxanthine–guanine phosphoribosyl transferase (*Hprt*) was used as a housekeeper gene (Table 3.). *Hprt* was chosen as an endogenous reference gene because it is a constitutively

expressed gene in the mammalian brain. Primers were designed with the help of Primer Express™ software (PE Applied Biosystems) to anneal to the different exons in order to avoid possible amplification of genomic DNA. The amount of the target gene was compared to the housekeeper gene in the experimental group and in the control group by means of the comparative ΔC_T method (Livak and Schmittgen 2001). The mRNA level in the saline-treated wild-type control group was defined as 1 and the increase of mRNA amounts were shown as the fold increase.

Table 3. Primer sequences used for qRT-PCR

Comt	Forward:	5'-GAGAAGGAGTGGGCCATGAA-3'
	Reverse:	5'-AACAAAGTCTGGCCTGTATCCAA-3'
Mao	Forward:	5'-GCCAAGAAGGATATATGGGTTCAA-3'
	Reverse:	5'-GAGCTGGAACATCCTTGGACT C-3'
TyHy	Forward:	5'-GCCAAGGACAAGCTCAGGAA-3'
	Reverse:	5'-CTTCACAGAGAATGGGCGCT-3'
Drd1	Forward:	5'-TCTCCTTCAAGCCCCTGGT-3'
	Reverse:	5'-GTCTCCCAGATCGGGCATT-3'
Drd2	Forward:	5'-TCCCAGCAGAAGGAGAAGAAA-3'
	Reverse:	5'-ACCAAGAACAATGGCAAGCAT-3'
Hprt	Forward:	5'-GCAGTACAGCCCCAAAATGG-3'
	Reverse:	5'-AACAAAGTCTGGCCTGTATCCAA-3'

4.6. Behavioural experiments (Papers I, II, III and IV)

The animals were brought into the experimental room one hour before the experiment. All behavioural experiments were performed between 11:00 a.m and 7:00 p.m. The motility and conditioned place preference tests were performed on separate animal groups. Since the behavioural experiments lasted for 6–8 h, precautions were taken to control possible daily fluctuations in the animals' locomotor activity. Therefore, the experiments were always performed in randomized order, that is, wild-type mice were always used in parallel with knockout animals.

4.6.1. Locomotor activity test (Papers I and II)

The locomotor activity of mice was studied in the photoelectric motility boxes (448 x 448 x 450 mm) connected to a computer (TSE Technical & Scientific Equipment GMBH). The illumination level in the transparent test boxes was ~400 lux. The apparatus-naïve mice were placed individually in the chambers, and vertical and horizontal activity was registered during a 30-min (I; II and III paper) or a 10-min (IV paper) observation period. After removing a mouse from the box the floor was cleaned by using 5 % alcohol solution. All the tests were carried out with different animal groups. Amphetamine was injected intraperitoneally 20 min; apomorphine, morphine, and naloxone 15 min before the measurement. The mice were pre-adapted to the motility boxes for three days before morphine (5 mg/kg; 10 mg/kg), an opioid receptor agonist was administered. The effect of naloxone (1 mg/kg; 10 mg/kg), an unselective opiate receptor antagonist, was studied both in mice pre-adapted and not adapted to the motility boxes (II paper). The locomotor effects of amphetamine (3 mg/kg and 6 mg/kg), increasing the release of catecholamines and apomorphine (0.1 mg/kg), an unselective agonist of dopamine D_1 and D_2, were studied in the animals not adapted to the test situation (I paper).

4.6.2. Behavioural sensitization (Papers III and IV)

The behavioural sensitization experiments were carried out in the photoelectric motility boxes (448 x 448 x 450 mm) connected to a computer (TSE Technical & Scientific Equipment GMBH). The illumination level of transparent test boxes was ~400 lux. All the animals were pre-adapted to the motility boxes and their motor activity was measured for three consecutive days before the sensitisation procedure was initiated. $CCK2R^{+/+}$ and $CCK2R^{-/-}$mice were randomly divided into treatment groups. In the first paper there was a saline-treated control group and amphetamine- (1 mg/kg) treated group (paper I). Immediately after the administration of saline or amphetamine the mice were placed into the motility box and their motor activity was recorded. In the sensitization experiment, described in the paper IV, the mice were at first administered vehicle or rimonabant (1 mg/kg) and then placed into their home cages. Thirty minutes later, the animals were administered saline or amphetamine (2 mg/kg) and then placed into the motility box and their motor activity was recorded for the subsequent 30 min. All the animals were treated for four consecutive days. The motor activity of mice on the first and fourth day of the experiment was compared in order to evaluate their motor sensitisation to amphetamine (1 or 2 mg/kg). In the sensitization experiment, described in the paper IV, after 21 days of withdrawal, all the mice were treated with amphetamine (2 mg/kg), and their motor activity was measured for the subsequent 30 min.

4.6.3. Place preference conditioning test (Paper III)

The place preference conditioning experiment were performed in three identical shuttle boxes (50 x 25 x 30). Each shuttle box was divided into two compartments of equal size by a sliding door having partition. The door allows free movement of a test animal between the two compartments if opened or restricts the movement of a mouse to the other compartment if closed. These compartments were distinguished by wall and floor colour (dark green versus pale green). The illumination level in the experiment room was ~200 lux. Each experiment consisted of pre-conditioning, conditioning, and post-conditioning periods. During the pre-conditioning period mice were placed three times into the apparatus for 15 minutes. The first and the second pre-conditioning tests were held on day 1. The third pre-conditioning test was held on day 2 of the experiment, and the time spent in the pale green compartment was measured. During the pre-conditioning and post-conditioning tests, the animals were allowed to explore both compartments freely. Each pre- and post-conditioning session began with an animal's initial placement into the dark green compartment. The shuttle boxes were cleaned carefully by 5% ethanol solution after each animal. The conditioning period consisted of 30-min experimental session performed twice a day for four consecutive days (days 3–6). Animals' movements were restricted into one compartment of the shuttle boxes. Every day animals received a saline injection as a first injection before being placed into one compartment and during the second session of the day, before their placement into the opposite compartment, they were injected with amphetamine (1 mg/kg; 2 mg/kg and 3 mg/kg). In the pre-conditioning session (days 1–2), the animals clearly preferred the dark green side and, therefore, the injections of amphetamine were paired with the pale green side avoided by mice during the pre-conditioning session (conditioning of place preference). The control group of animals received their injection of saline in both compartments. Injections were given immediately before the conditioning sessions. Fifteen-minute post-conditioning tests were performed on day 7 of the experiments and the time spent in the pale green side of the shuttle box was measured.

4.7. Statistics

The results have been expressed as mean values ± S.E.M. The behavioural and gene expression studies were analysed by using analysis of variance (ANOVA) or multivariate analysis of variance (MANOVA). *Post hoc* comparisons between means of the individual groups were performed by Tukey HSD or Newman-Keuls test (Statistica for Windows software). The student's t-test was applied for the analysis of radioligand binding data.

5. RESULTS

5.1. Locomotor activity test (Papers I and II)

5.1.1. Effect of apomorphine (Paper I)

The administration of a low dose of apomorphine (0.1 mg/kg), an unselective agonist of DA receptors, reduced the locomotor activity [one-way ANOVA: distance in locomotion $F_{5,42}$=10.88, P<0.01,). The application of two-way ANOVA did not reveal any statistical differences when the genotype and treatment data were compared (Paper I). However, in mutant animals, the motor suppressant effect of apomorphine was stronger than in the wild-type mice, since the DA agonist reduced not only the distance travelled and corner entries but also the number of rearing (one-way ANOVA: $F_{5,42}$=5.87, P<0.01) (Figure 2).

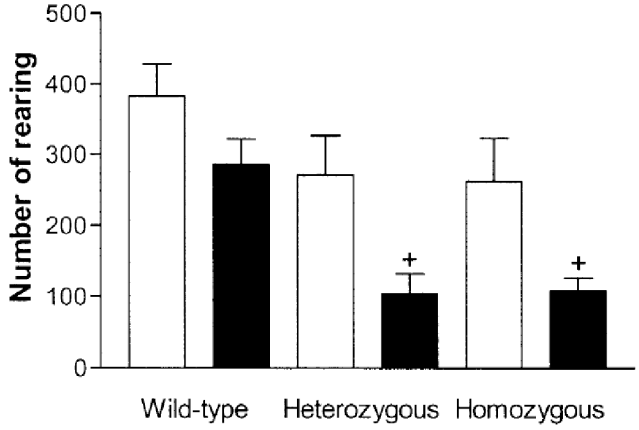

Figure 2. Apomorphine (0.1 mg/kg) induced motor suppression in cholecystokinin CCK2R receptor-deficient mice. The number of animals in each group was 8. *White bars* saline, *black bars* apomorphine. +P<0.05 (compared with the respective saline-treated group; Tukey HSD test after significant one-way analysis of variance)

5.1.2. Effect of amphetamine (Paper I)

The applying of two-way ANOVA (genotype; treatment) established statistically significant differences when the effect of amphetamine (3 mg/kg and 6 mg/kg) was compared between the wild-type CCK2R$^{+/+}$ and the CCK2R$^{-/-}$ mice (ANOVA: distance in locomotion $F_{4,117}$=5.85, P<0.01; $F_{4,117}$=2.90, P<0.05). The administration of amphetamine (3 mg/kg and 6 mg/kg) to the CCK2R$^{+/+}$ mice induced a dose-dependent increase in locomotor activity (Figure 3.). A lower dose of amphetamine (3 mg/kg) slightly, but insignificantly, increased the locomotion in the CCK2R$^{+/-}$ mice, but not in the homozygous CCK2R$^{-/-}$ animals. The higher dose of amphetamine (6 mg/kg)

caused a significant increase in the locomotor activity in mutant mice. In the CCK2R$^{+/-}$ heterozygous mice, the effect of amphetamine (6 mg/kg) did not differ from that in the wild-type CCK2R$^{+/+}$ littermates; whereas, in the homozygous CCK2R$^{-/-}$ mice, the higher dose of amphetamine caused the significantly stronger effect (Paper I).

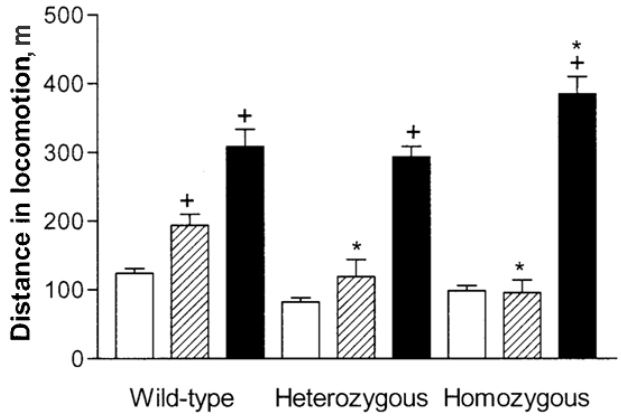

Figure 3. Amphetamine (3 and 6 mg/kg) induced motor stimulation in cholecystokinin (CCK)2R deficient mice. The number of animals in each group was 14. *White bars* saline, *striped bars* amphetamine 3 mg/kg, *black bars* amphetamine 6 mg/kg. +P<0.05 (compared with the respective saline-treated group; Tukey HSD test after two-way analysis of variance), *P<0.05 (compared with the respective wild type group).

5.1.3. Locomotor adaptation (Paper II)

In the first adaptation session the locomotor activity of mutant mice did not differ from that of wild-type littermates (Figure 4.). Only the number of rearing was somewhat higher in homozygous CCK2R$^{-/-}$ animals. However, during the third adaptation session some obvious differences became evident. There was a significant reduction of locomotor activity in the wild-type mice but not in the homozygous CCK2R$^{-/-}$ mice. The wild-type animals displayed a significant reduction of time in locomotion (two-way ANOVA: genotype $F_{1,28}$=10.43, P<0.01; experiment $F_{1,28}$=20.1, P<0.01; genotype × experiment $F_{1,28}$=7.46, P<0.01), distance travelled (two-way ANOVA: genotype $F_{1,28}$=5.91, P<0.05; experiment $F_{1,28}$=20.1, P <0.01; genotype × experiment $F_{1,28}$=6.56, P<0.05) and the number of corner entries (two-way ANOVA: genotype $F_{1,28}$=1.36, P=0.25; experiment $F_{1,28}$=19.3, P<0.01; genotype × experiment $F_{1,28}$=5.15, P<0.05) compared with the homozygous CCK2R$^{-/-}$ animals (Figure 4.). The application of two-way ANOVA did not establish any significant differences in the frequency of rearing (genotype $F_{1,28}$=25.17, P<0.01; experiment $F_{1,28}$=5.17, P<0.05; genotype × experiment $F_{1,28}$=2.55, P =0.12).

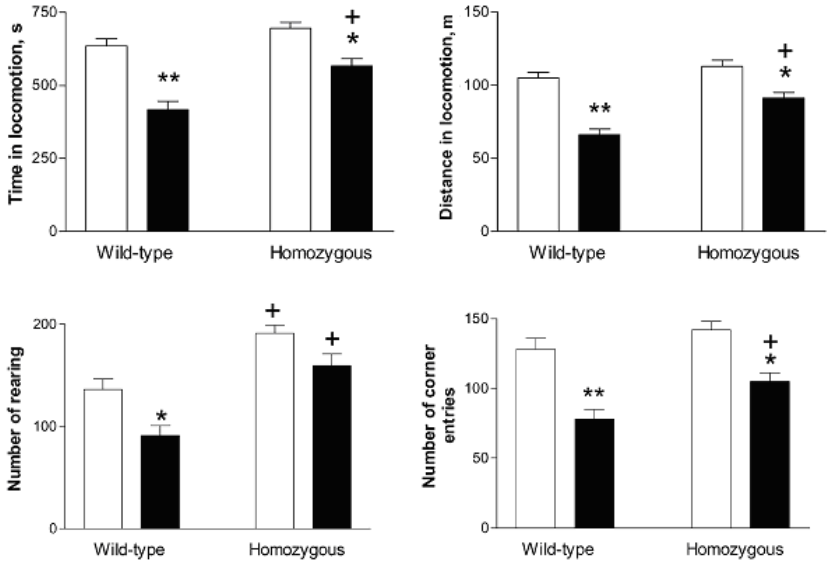

Figure 4. Different adaptation of wild-type mice and mice without CCK2R in the motility boxes. The number of animals in each group was 25. *White bars* – the motor activity of mice during the first experiment, *black bars* – the response of animals during the third experiment. (*) $P < 0.05$; (**) $P < 0.001$ (compared with the first experiment, Tukey HSD test after the significant two-way ANOVA); (+) $P < 0.05$ (compared with the respective group of wild-type mice).

5.1.4. Effect of morphine (Paper II)

The administration of morphine (5–10 mg/kg) induced strong motor stimulation in the wild-type CCK2R$^{+/+}$ mice habituated to the motility boxes (Figure 5.). This effect was not established in the homozygous CCK2R$^{-/-}$ animals habituated to the experimental environment. A lower dose (5 mg/kg) of morphine did not affect markedly the motor activity of the wild-type CCK2R$^{+/+}$ mice, whereas a higher dose (10 mg/kg) increased the time in locomotion (two-way ANOVA: genotype $F_{1,42}$=0.39, p=0.39; treatment $F_{2,42}$=11.1, P<0.01; genotype × treatment $F_{2,42}$=5.40, p<0.01), the distance travelled (two-way ANOVA: genotype $F_{1,42}$=0.17, P =0.90; treatment $F_{2,42}$=14.8, P<0.01; genotype × treatment $F_{2,42}$=4.29, P<0.05), the number of rearing (two-way ANOVA: genotype $F_{1,42}$=1.70, P=0.20; treatment $F_{2,42}$=6.7, P<0.01; genotype × treatment $F_{2,42}$=4.81, P<0.05) and the frequency of corner entries (two-way ANOVA: genotype $F_{1,42}$=1.19, P=0.28; treatment $F_{2,42}$=19.0, P<0.01; genotype × treatment $F_{2,42}$=3.28, P <0.05).

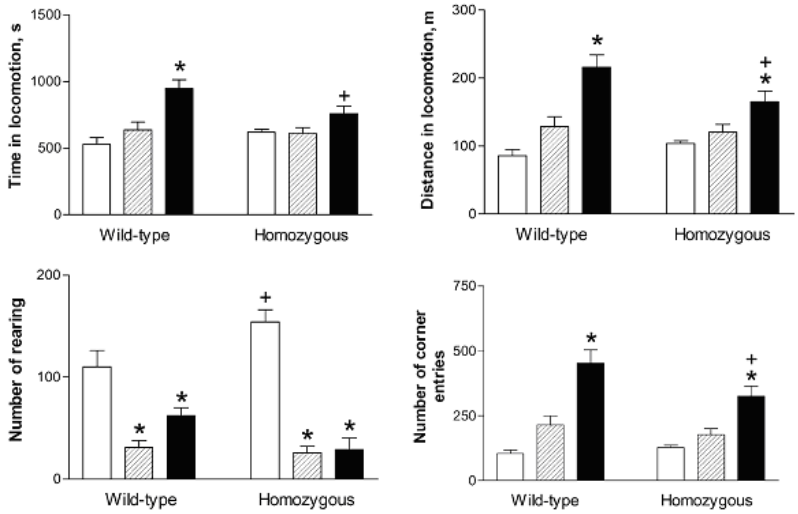

Figure 5. The effect of morphine (5 and 10 mg/kg i.p.) on the locomotor activity of mice without CCK2 receptors. The number of animals in each group was 17. *White bars* – saline treatment, *striped bars* – morphine (5 mg/kg), *black bars* – morphine (10 mg/kg). (*) $P < 0.05$ (compared with the respective saline-treated group, Tukey HSD test after the significant two-way ANOVA); (+) $P < 0.05$ (compared with the respective group of wild-type mice).

5.1.5. Effect of naloxone (Paper II)

The administration of opioid receptor antagonist naloxone (1 and 10 mg/kg) to non-habituated mice induced a dose-dependent reduction of the locomotor activity in the wild-type CCK2R$^{+/+}$ and homozygous CCK2R$^{-/-}$ mice (Figure 6). However, the application of two-way ANOVA did not reveal any significant differences if the action of naloxone was compared in the wild-type CCK2R$^{+/+}$ and mutant animals. Naloxone induced a decrease of time in locomotion (two-way ANOVA: genotype: $F_{1,42}$=0.95, P=0.76; treatment: $F_{2,42}$=8.91, P<0.01; genotype × treatment: $F_{2,42}$=0.87, P=0.42), distance travelled (two-way ANOVA: genotype: $F_{1,42}$=0.09, P=0.77; treatment: $F_{2,42}$=12.13, P<0.01; genotype × treatment: $F_{2,42}$=1.21, P=0.30), number of rearing (two-way ANOVA: genotype: $F_{1,42}$=7.94, P<0.01; treatment: $F_{2,42}$=6.44, P<0.01; genotype × treatment: $F_{2,42}$=1.06, P=0.35) and frequency of corner entries (two-way ANOVA: genotype: $F_{1,42}$=3.05, P=0.09; treatment: $F_{2,42}$=12.6, P<0.01; genotype × treatment: $F_{2,42}$=1.79, P=0.17). Nonetheless, the usage of post-hoc analysis demonstrated that the administration of naloxone (1 and 10 mg/kg) inhibited the increased frequency of rearing in the homozygous CCK2R$^{-/-}$ mice. Also, the highest dose of naloxone (10 mg/kg) did not induce a significant reduction of time in locomotion in the homozygous mice CCK2R$^{-/-}$ (Tukey HSD test: wild-type CCK2R$^{+/+}$ mice P<0.05 and homozygous CCK2R$^{-/-}$ animals P=0.11) (Figure 6.).

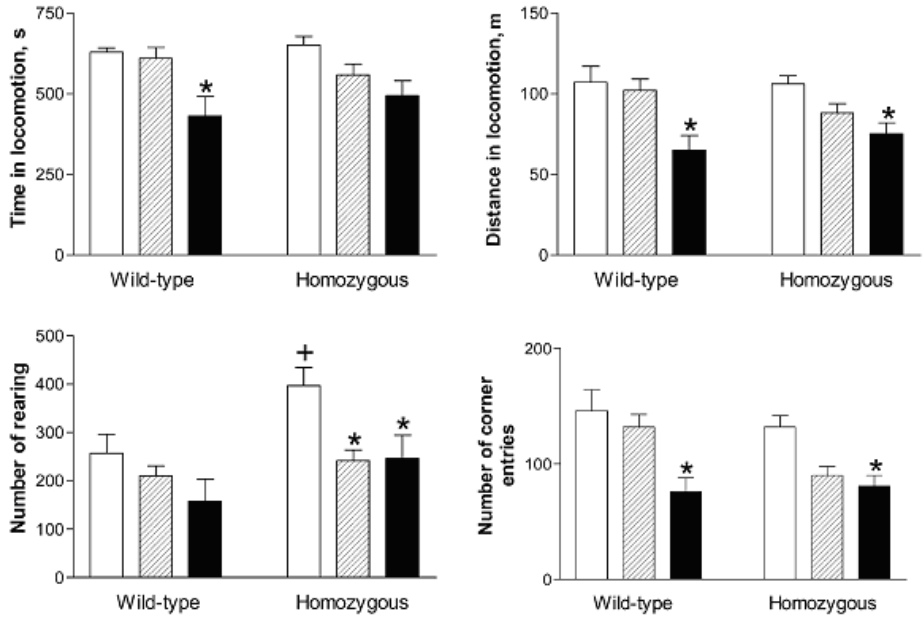

Figure 6. The effect of naloxone (1 and 10 mg/kg i.p.) on the locomotor activity of CCK2 receptor-deficient mice not adapted to the motility boxes. The number of animals in each group was eight. *White bars* – saline treatment, *striped bars* – naloxone (1 mg/kg), *black bars* – naloxone (10 mg/kg). (*) $P < 0.05$ (compared with the saline-treated mice, Tukey HSD test after the significant one-way ANOVA); (+) $P < 0.05$ (compared with the saline-treated wild type mice).

In mice, habituated to the experimental environment, the application of two-way ANOVA did not distinguish the action of naloxone in the wild-type CCK2R$^{+/+}$ and homozygous CCK2R$^{-/-}$ mice (Figure 7.) (time spent in locomotion (two-way ANOVA: genotype: $F_{1,52}$=0.27, P=0.61; treatment: $F_{2,52}$=4.06, P<0.05; genotype × treatment: $F_{2,52}$=1.41, P=0.25); distance travelled (two-way ANOVA: genotype: $F_{1,52}$=0.48, P=0.49; treatment: $F_{2,52}$=6.44, P<0.01; genotype × treatment: $F_{2,52}$=2.26, P=0.11); frequency of rearing (two-way ANOVA: genotype: $F_{1,52}$=0.44, P=0.51; treatment: $F_{2,52}$=1.08, P=0.35; genotype × treatment: $F_{2,52}$=0.77, P=0.47) and number of corner entries (two-way ANOVA: genotype: $F_{1,52}$=0.38, P=0.54; treatment: $F_{2,52}$=5.77, P<0.01; genotype × treatment: $F_{2,52}$=1.06, P=0.35)). However, the situation was different after the *post-hoc* analysis. Naloxone (10 mg/kg) induced the inhibition of locomotor activity only in the homozygous CCK2R$^{-/-}$, but not in the wild-type CCK2R$^{+/+}$ mice (Tukey HSD test: time in locomotion, P<0.05; distance travelled, P<0.01, number of corner entries P<0.05) (Figure 7.).

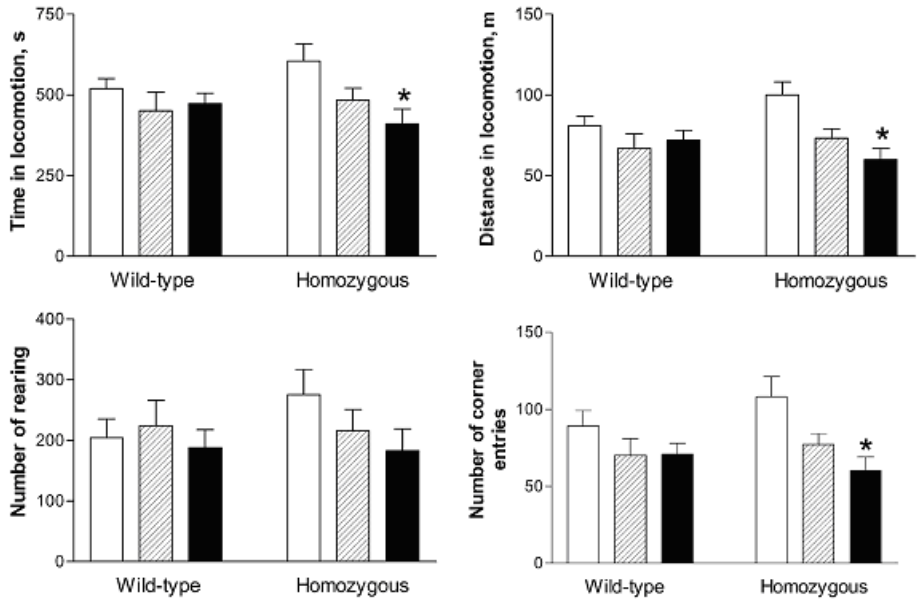

Figure 7. The effect of naloxone (1 and 10 mg/kg i.p.) on the locomotor activity of the CCK2 receptor-deficient mice adapted to the motility boxes. The number of animals in each group was 9–10. *White bars* – saline treatment, *striped bars* – naloxone (1 mg/kg), *black bars* – naloxone (10 mg/kg). (*) $P < 0.05$ (compared with saline-treated homozygous mice, Tukey HSD test after the significant one-way ANOVA).

5.2. Behavioural sensitization (Papers III and IV)

5.2.1. Behavioural sensitization with amphetamine (Paper III)

A four-day treatment with saline did not affect the locomotor activity of the homozygous and wild-type animals (Figure 8). However, the repeated treatment with amphetamine (1 mg/kg) increased the locomotor activity of the mice compared to saline treatment. Although, the application of repeated measure MANOVA did not reveal any significant genotypic differences in action of amphetamine (total distance, MANOVA: genotype: $F_{(1,49)}$=0.04, P=0.837; treatment: $F_{(1,49)}$=0.89, P=0.349; day: $F_{(1,49)}$=16.67, P<0.001; genotype × treatment: $F_{(1,49)}$=0.86, P=0.36; genotype × day: $F_{(1,49)}$=0,55, P=0.464; treatment × day: $F_{(1,49)}$=15.78, P<0.001; genotype × treatment × day: $F_{(1,49)}$=0.84, P=0.365); (number of corner visits: MANOVA: genotype: $F_{(1,49)}$=0.05, P=0.825; treatment: $F_{(1,49)}$=6.78, P=0.012; day: $F_{(1,49)}$=17.00, P<0.001; genotype × treatment: $F_{(1,49)}$=0.99, P=0.324; genotype × day: $F_{(1,49)}$=1.14, P=0.292; treatment × day: $F_{(1,49)}$=25.38, P<0.001; genotype × treatment × day: $F_{(1,49)}$=0.29, P=0.593), the situation was a bit different after the *post hoc* analysis. The application of *post hoc* analysis revealed that the distance travelled was significantly increased in the homozygous mice, but not in the

wild-type animals. Taking into account the actual numbers, one can find that in the case of the wild-type mice the increase in distance travelled was only 15 m, whereas in the homozygous animals the respective value was 38 m. This is almost a 2.5-fold difference in the action of amphetamine if the response of the wild-type and homozygous animals is compared. The repeated amphetamine treatment, 1 mg/kg only tends to increase the total distance of movement of the wild-type mice, whereas in the knockout mice this dose caused a significant increase in this parameter of the locomotor activity by the forth administration if compared with the saline treated group of the same genotype (Newman-Keuls test, P <0.05). Also, the frequency of corner entries was more prominently increased in the homozygous mice compared to their wild-type littermates. In comparison with the saline treated animals, only the number of corner visits of the amphetamine treated wild-type mice was significantly grown by the forth day (Newman-Keuls test, P<0.01). At the same time evoked the forth amphetamine administration in the knockout mice group a highly significant increase in the number of corner entries (Newman-Keuls test, P<0.001) (paper III).

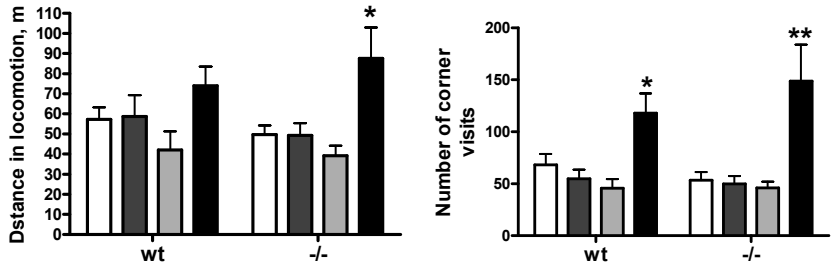

Figure 8. Effect of repeated treatment of amphetamine (1 mg/kg) on the locomotor activity of the wild-type (wt) and homozygous (−/−) CCK2 receptors deficient mice. The number of mice in the saline-treated group was 13 and in the amphetamine-treated group 14. *White bars*: acute saline treatment; *dark grey* bars: 4-day saline treatment; *pale grey* bars: acute amphetamine treatment; *black bars*: 4-day amphetamine treatment. **P* < 0.05; ***P* < 0.001 (compared with the repeated saline treatment of respective genotype, Newman–Keuls test after the significant three-way ANOVA).

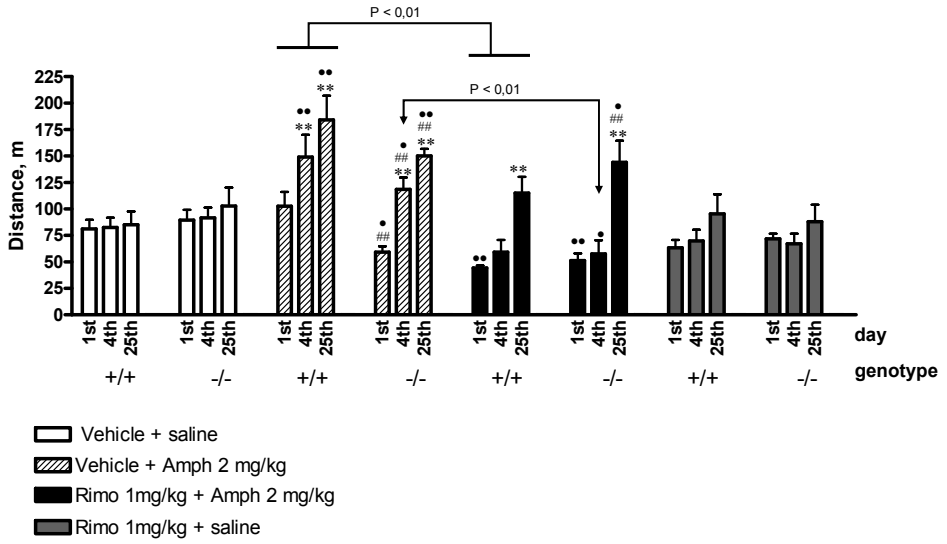

Figure 9. Effect of rimonabant (1 mg/kg) on motor sensitisation to amphetamine (2 mg/kg). On days 1–4, vehicle or rimonabant and saline or amphetamine were injected 30 and 0 min before testing, respectively. On day 25, amphetamine was injected immediately before testing.
(•) $P < 0.05$; (••) $P < 0.01$ compared to the respective control group treated with vehicle and saline on days 1 - 4; Newman-Keuls test.
(**) $P < 0.01$ compared to the 1st treatment day of the same group; Newman-Keuls test.
(#) $P < 0.05$; (##) $P < 0.01$ compared to wild-type mice; Newman-Keuls test. N = 7–9 mice per group.
Effect of four consecutive treatments (1st day compared with 4th day). Three-way ANOVA with repeated measures. Genotype: $F_{(1,56)} = 0.62$, $P = 0.44$; treatment: $F_{(3,56)} = 11.99$, $P < 0.01$; measurement: $F_{(1,56)} = 27.21$, $P < 0.01$; genotype × treatment: $F_{(3,56)} = 2.51$, $P = 0.17$; genotype × measurement: $F_{(1,56)}=0.11$, $P=0.75$; treatment × measurement: $F_{(3,56)}=14.81$, $P<0.01$) genotype × treatment × measurement: $F_{(3,56)} = 0.74$, $P = 0.53$).
Effect of amphetamine (2 mg/kg) after withdrawal (1st day compared with 25th day). Three-way ANOVA with repeated measures. Genotype: $F_{(1,56)} = 0.04$, $P = 0.85$; treatment: $F_{(3,56)} = 6.06$, $P < 0.01$; measurement: $F_{(1,56)} = 108.75$, $P < 0.01$; genotype × treatment: $F_{(3,56)} = 2.57$, $P = 0.06$; genotype × measurement: $F_{(1,56)} = 0.33$, $P = 0.57$; treatment × measurement: $F_{(3,56)} = 16.44$, $P < 0.01$; genotype × treatment × measurement: $F_{(3,56)} = 0.78$, $P = 0.51$).

5.2.2. Behavioural sensitization with amphetamine and the effect of rimonabant co-treatment (Paper IV)

In the behavioural sensitisation test, the first administration of amphetamine (2 mg/kg) suppressed motor activity in the $CCK2R^{-/-}$ mice but not in the $CCK2R^{+/+}$ mice. The ANOVA results have been given in the legend of Figure 9. Four subsequent amphetamine treatments induced a significant motor sensiti-

sation in both the CCK2R$^{-/-}$ and CCK2R$^{+/+}$ mice. However, in the CCK2R$^{-/-}$ mice receiving amphetamine, their motor activity measured on day 4 was less pronounced. Nevertheless, the effect of amphetamine was significantly stronger on the fourth day in these mice when compared to their first treatment with amphetamine (P=0.0001) and treatment with saline (P=0.0129) on the fourth day in the CCK2R$^{-/-}$ mice (Figure 9). Sensitisation to amphetamine was confirmed by injecting amphetamine 21 days after the last experiment. Both, the CCK2R$^{-/-}$ and CCK2R$^{+/+}$ animals that had received amphetamine treatment for 4 days, displayed significant motor sensitisation compared to their first treatment session and compared to the animals that were acutely treated with amphetamin after having previously received saline. Similar to day 4, the amphetamine-induced motor effect was weaker in the CCK2R$^{-/-}$ mice. Pre-treatment with the CB1 receptor antagonist rimonabant at the dose of 1 mg/kg did not affect locomotion when given alone but inhibited the effect of amphetamine. The co-administration of rimonabant and amphetamine for 4 days completely blocked the stimulant effect of the dopamine agonist. When amphetamine was injected 21 days later, the CCK2R$^{+/+}$ animals that were previously administered rimonabant and amphetamine did display motor sensitisation, but this sensitisation was significantly less pronounced than in the group receiving vehicle and amphetamine. Remarkably, in the case of the CCK2R$^{-/-}$ animals, previous co-administration of rimonobant with amphetamine did not affect the development of sensitisation to amphetamine (Paper IV).

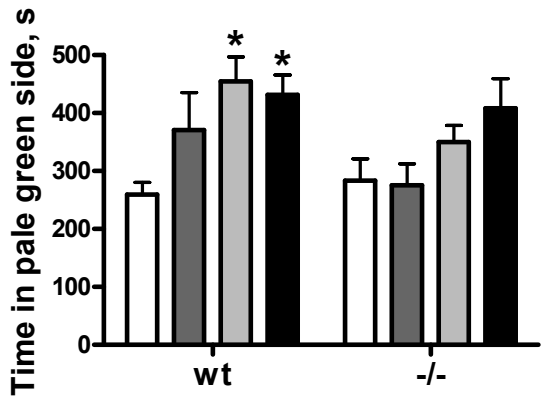

Figure 10. Effect of amphetamine (1, 2 and 3 mg/kg) on the place preference of the wild-type (wt) and the homozygous (−/−) the CCK2 receptors deficient mice. *White bars*: saline (14 wild-type and 13 homozygous mice); *dark grey* bars: amphetamine 1 mg/kg (9 wild-type and 8 homozygous mice); *pale grey* bars: amphetamine 2 mg/kg (7 wild-type and 8 homozygous mice); *black bars*: amphetamine 3 mg/kg (8 wild-type and 10 homozygous mice). *$P < 0.05$ (compared with the saline-treated wild-type mice, Newman–Keuls test after the significant two-way ANOVA).

5.3. Conditioned place preference with amphetamine (Paper III)

In the conditioned place preference test during the pre-conditioning period both the wild-type and homozygous mice preferred to stay in the dark green compartment of the shuttle box (ANOVA: genotype: $F_{(1,75)}$=0.067, P=0.798) (Figure 10). The pairing of pale green part with amphetamine injections significantly increased the preference of mice for this compartment (two-way ANOVA: genotype: $F_{(1,69)}$=2.87, P=0.095; treatment: $F_{(1,69)}$=6.34, P<0.001; genotype × treatment: $F_{(1,69)}$=1.19, P=0.320). The application of *post hoc* analysis revealed that amphetamine induced a significant place conditioning in the wild-type mice, but not in the homozygous animals. Already 1 mg/kg of amphetamine tended to shift the preference of the wild-type mice into the pale green side and at the doses of 2 and 3 mg/kg induced a significant change in the place preference (Newman-Keuls test, P<0.05). Amphetamine caused the strongest effect at the dose of 2 mg/kg in the wild-type mice. Amphetamine increased the time spent in the not-preferred side for 195 s in these animals. The respective difference for the homozygous mice was only 66 s. This demonstrates almost a three-fold difference in the action of the drug. By contrast, in the homozygous mice even the highest dose of amphetamine (3 mg/kg) did not cause a significant shift (Newman-Keuls test, P=0.158) (Figure 10.). The pairing of the dark and pale green box with saline injections did not change the animals' place preference compared to the pre-conditioning session (Paper III).

5.4. Content of dopamine and its metabolites in the brain structures (Paper I)

The assay of dopamine and its metabolite levels in the cerebral cortex, the striatum and the hypothalamus did not reveal significant differences between the $CCK2R^{+/+}$ and $CCK2R^{-/-}$ mice (Table 4.). The number of mice in both genotype group was three.

Table 4. Content of dopamine and its metabolites in the brain structures (pmol/mg wet weight tissue).

	Cerebral cortex		Striatum		Hypotalamus	
	+/+	-/-	+/+	-/-	+/+	-/-
Dopamine	0.32±0.12	0.24±0.11	65.5±6.0	53.2±3.5	1.3±0.3	1.2±0.2
DOPAC	0.42±0.06	0.30±0.10	4.7±0.2	5.4±0.8	0.4±0.03	0.43±0.01
HVA	0.32±0.06	0.35±0.11	7.1±0.7	6.1±1.3	1.5±0.2	1.4±0.1

5.5. Radioligand binding data (Papers I an II)

5.5.1. [^{3}H]-spiperone binding in the brain (Paper I)

The density of [^{3}H]-spiperone binding sites was significantly higher in the striatum of the homozygous CCK2R$^{-/-}$ mice than their wild-type CCK2R$^{+/+}$ littermates (Figure 11.). Also, in the mesolimbic structures, the number of [^{3}H]-spiperone binding sites was somewhat increased (not statistically significant) in the homozygous CCK2R$^{-/-}$ mice. A similar trend of increased [^{3}H]-spiperone binding was apparent in the striatum of the heterozygous CCK2R$^{+/-}$ animals. In the cerebral cortex, we did not find any differences between the wild type CCK2R$^{+/+}$and the mutant mice.

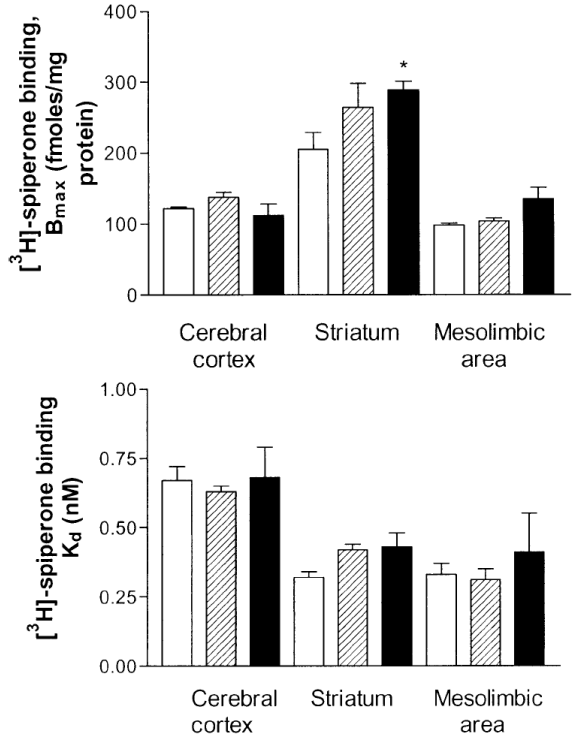

Figure 11. The parameters of [^{3}H]-spiperone binding in the brain structures of the CCK_2 receptor-deficient mice. The number of animals in each group was 18, the brains of 6 mice were pooled, and the mean is the result of three experiments. *White bars* wild-type, *striped bars* heterozygous, *black bars* homozygous. *P<0.05 (compared with the wild-type group, student's *t*-test)

5.5.2. [^{3}H]-diprenorphine binding in the brain (Paper II)

The radioligand binding studies with [^{3}H]-diprenorphine demonstrated an increase in the affinity of the opioid binding sites in the cerebral cortex of the mutant mice compared to the wild-type CCK2R$^{+/+}$ littermates (Figure 12.). However, the decreased affinity and increased density of the opioid binding sites was established in the striatum of the homozygous CCK2R$^{-/-}$ mice. The parameters of [^{3}H]-diprenorphine binding were not affected in the mesolimbic structures and the hippocampus.

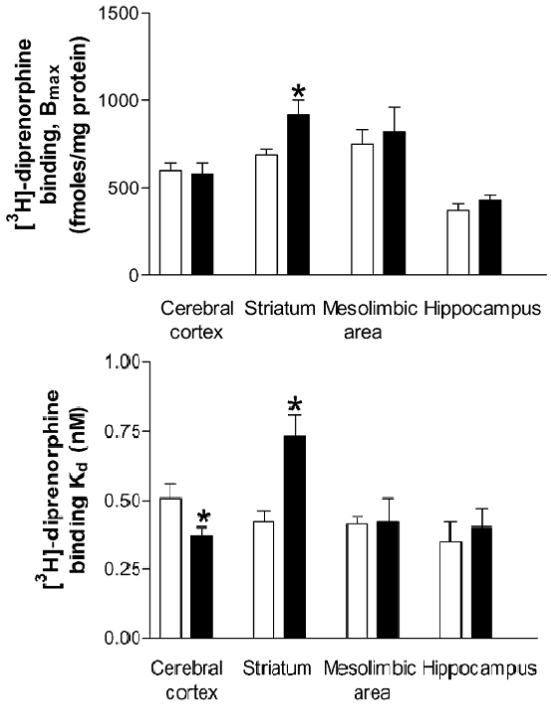

Figure 12. The parameters of [^{3}H]-diprenorphine binding in the brain structures of the mice without CCK2 receptors. The number of animals in each group was 24, the brains of six mice were pooled, and the mean is the result of four experiments. *White bars* – wild type, *black bars* – homozygous. (*) $P < 0.05$ (compared with the wild-type group, Student's *t*-test).

5.6. Gene expression analysis (Paper III)

We established a genotype-dependent difference in expression of *Drd2* gene in the mesencephalon (Figure 13. A.). (two-way ANOVA: genotype: $F_{(1,12)} = 10.61$, $P = 0.007$; treatment: $F_{(1,12)} = 0.47$, $P = 0.507$; genotype × treatment: $F_{(1,12)} = 1.6$, $P = 0.229$). Indeed, the expression of *Drd2* gene in the saline-treated homozygous CCK2R$^{-/-}$ mice was significantly lower in comparison with

the wild-type animals (Newman-Keuls test, $P<0.05$). Four-day administration of amphetamine induced changes in the expression of *Comt* gene (two-way ANOVA: genotype: $F_{(1,12)} = 0.59$, $P = 0.46$; treatment: $F_{(1,12)} = 16.2$, $P < 0.001$; genotype × treatment: $F_{(1,12)} = 0.65$, $P = 0.44$) and *Tyhy* gene (two-way ANOVA: genotype: $F_{(1,12)} = 0.09$, $P = 0.77$; treatment: $F_{(1,12)} = 49.2$, $P < 0.001$; genotype × treatment: $F_{(1,12)} = 0.01$, $P = 0.93$). The expression level of *Tyhy* and *Comt* genes in the amphetamine-sensitized mice of both genotypes was significantly higher compared to the respective groups of the saline-treated mice (Newman-Keuls tests, $P<0.001$ and $P<0.05$, respectively).

In the striatum, we established a genotype-dependent effect of amphetamine treatment on the expression of *Tyhy* (Figure 13. B.) (two-way ANOVA: genotype: $F_{(1,12)} = 3.22$, $P = 0.11$; treatment: $F_{(1,12)} = 0.55$, $P = 0.48$; genotype × treatment: $F_{(1,12)} = 14.8$, $P = 0.005$) and *Drd1* (two-way ANOVA: genotype: $F_{(1,12)} = 2.36$, $P = 0.16$; treatment: $F_{(1,12)} = 2.53$, $P = 0.15$; genotype × treatment: $F_{(1,12)} = 17.8$, $P = 0.003$) genes. *Tyhy* and *Drd1* mRNA levels were significantly elevated only in the amphetamine-sensitized wild-type mice. By contrast, the level of these genes (Newman-Keuls test: *Tyhy* gene $P<0.05$; *Drd1* gene $P<0.01$) was markedly lower in the amphetamine-treated homozygous $CCK2R^{-/-}$ mice compared with the corresponding group of the wild-type animals.

In the mesolimbic structures, we found treatment- and genotype-dependent changes of *Tyhy* (Figure 13. C.) (two-way ANOVA: genotype: $F_{(1,12)} = 5.48$, $P = 0.047$; treatment: $F_{(1,12)} = 20.0$, $P = 0.002$; genotype × treatment; $F_{(1,12)} = 1.73$, $P = 0.23$) and *Drd2* (two-way ANOVA: genotype: $F_{(1,12)} = 24.3$, $P = 0.001$; treatment: $F_{(1,12)} = 6.36$, $P = 0.036$; genotype × treatment: $F_{(1,12)} = 1.39$, $P = 0.27$) gene expression. In this particular brain region *Tyhy* gene expression was significantly higher in the saline-treated homozygous $CCK2R^{-/-}$ mice compared to their wild-type littermates (Newman-Keuls test, $P<0.05$). 4-day treatment with amphetamine in the wild-type mice induced a significant increase in the expression of *Tyhy* and *Drd2* genes. At the same time, the expression level of *Drd2* gene in the amphetamine-treated homozygous $CCK2R^{-/-}$ mice was significantly lower compared to the respective group of the wild-type animals (Newman-Keuls test, $P<0.01$) (Paper III).

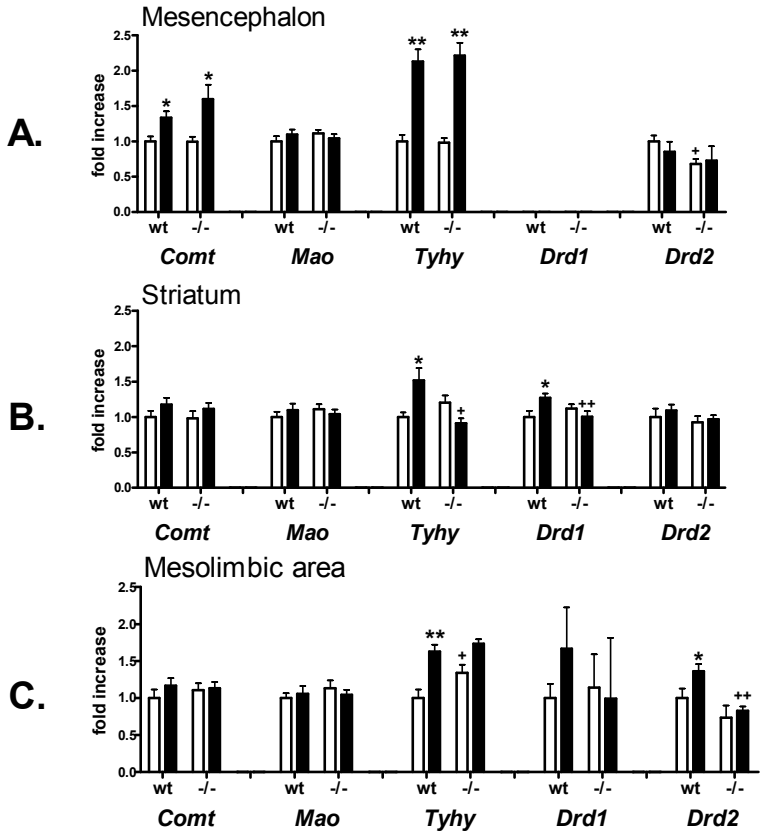

Figure 13. Effect of the repeated amphetamine (1 mg/kg) administration on the gene expression levels of the dopamine-related genes in the mesencephalon (A.), striatum (B.) and mesolimbic (C.) structures of the wild-type (wt) and homozygous (−/−) CCK2 receptor deficient mice. Every experimental group (n = 13) was pooled as follows: three pools including samples from three mice and one pool including samples from four animals. The number of independent samples in each case was four. *White bars*: repeated saline treatment; *black bars*: repeated amphetamine treatment. *P < 0.05; **P < 0.01 (compared with the saline treated group of respective genotype, Newman–Keuls procedure after the significant ANOVA); (+) P < 0.05; (++) P < 0.01 (compared with the respective treatment of the wild-type group).

6. DISCUSSION

6.1. Alterations in dopamine mechanisms due to the lack of CCK2R

The genetic deletion of CCK2R causes alterations in the functions of the central dopaminergic system. The effects of two dopaminergic drugs, apomorphine, an unselective D_1 and D_2 receptor agonist, and amphetamine, a drug increasing the release of dopamine (DA) and noradrenaline from the presynaptic terminals (Fleckenstein et al. 2007), were studied in CCK2R deficient mice (Paper I). The treatment with apomorphine 0.1 mg/kg, reduces the motor activity of mice. The motor suppressant effect of apomorphine is believed to be caused by the stimulation of presynaptic somato-dendritic D_2 auto-receptors located on the DA neurones (Meltzer 1980). The activation of dopamine autoreceptors located at the soma decreases the firing rate of dopaminergic neurons (Bunney et al. 1973) and axon-terminal autoreceptors inhibit DA release (Cubeddu and Hoffmann 1982). In rats the systemic treatment with apomorphine induces suppression of cell firing in the VTA (Bortolozzi et al. 2007). The motor depressant effect of apomorphine 0.1 mg/kg was apparently stronger in the heterozygous $CCK2R^{+/-}$ and the homozygous $CCK2R^{-/-}$ receptor-deficient mice than their $CCK2R^{+/+}$ littermates. The increased response is probably related to the increased sensitivity of the presynaptic DA receptors. This is in good agreement with some earlier pharmacological studies in which the pre-treatment of mice with the CCK2R antagonist L-365,260 potentiated the motor suppressant action of apomorphine (Vasar et al. 1991). Moreover, electrophysiological studies in rats demonstrated that the acute and long-term administration of LY262691 and related pyrazolidinone CCK2R receptor antagonists decreased the number of spontaneously active DA cells in the midbrain structures, probably via action in the nucleus accumbens and the prefrontal cortex (Rasmussen et al. 1993). Also, it is proved that CCK2R stimulation functionally opposes the postsynaptic effects of DA in the nucleus accumbens (Weiss et al. 1989; Yim and Mogenson 1991). The administration of amphetamine (3 mg/kg and 6 mg/kg) induced a dose-dependent motor stimulation in $CCK2R^{+/+}$. However, in the mutant mice, the effect of amphetamine was different. In the heterozygous $CCK2R^{+/-}$ mice 3 mg/kg tended to increase their motor activity, but 6 mg/kg had the same effect as in the $CCK2R^{+/+}$ mice. In the homozygous $CCK2R^{-/-}$ mice, the 3 mg/kg did not affect parameters of the horizontal locomotor activity. The treatment with the dose of 6 mg/kg induced a significant increase in their locomotor activity. It should be noted that the effect of the dose of 6 mg/kg in the $CCK2R^{-/-}$ mice on these parameters was significantly higher from that seen in the heterozygous $CCK2R^{+/-}$ and wild-type animals. The increased response to amphetamine 6 mg/kg in the homozygous $CCK2R^{-/-}$ animals could be related to the increased activity of the postsynaptic DA receptors. Previous studies seem to support this hypothesis. The administration of CCK into the anterior NAc inhibits DA-induced hyperlocomotion, and this

effect is mediated via CCK2R (Crawley 1992; Dauge et al. 1990). However, the blockade of CCK2R in the NAc increases amphetamine-induced DA release and hyperlocomotion (Altar and Boyar 1989). Centrally administrated CCK2R agonists (BOC-CCK-4 or CCK-8U) attenuated DA release. To confirm an arisen idea about the altered activity of the mesolimbic dopaminergic neurons the content of DA and metasbolites in the cerebral cortex, the striatum and the hypothalamus, was measured. Nevertheless, despite the obvious dopamine related behavioural changes, the difference in the content of DA and metabolites between the $CCK2R^{+/+}$ and $CCK2R^{-/-}$ mice brain samples, was not detectable (Paper I). However, in the radioligand binding study on the crude striatal and mesolimbic brain samples the increased density of the D_2 receptors becomes evident. Earlier biochemical studies showed that CCK-8S through the activation of CCK2R decreases the affinity to D_2 receptor agonists and special effects of the pre- and postsynaptic D_2 receptors in the medial posterior-nucleus accumbens and caudate putamen (Dasgupta et al. 1996; Ferraro et al. 1996; Li et al. 1995). Our striatal preparations consisted mainly of dorsal striatal caudate-putamen structures which are innervated by the primary motor cortex, the anterior premotor and cingulated areas, the substantia nigra pars compacta (subNc); and in turn projects to the globus pallidus, and the substantia nigra pars reticulata (subNr). The main structure in the mesolimbic preparations was NAc, which receives inputs from the mPFC, hippocampus, amygdala and VTA; and sends projections to the VP, the subNc, the VTA and the hypothalamus. The dorsal striatum is mainly related to the voluntary motor function, whereas the NAc is mostly an interface between the limbic and the motor system and plays a major role in motivated and goal directed behaviours (David et al. 2005) In the [^{3}H] spiperone binding assay, we detected significantly increased density of the D_2 receptors in the striatum of homozygous $CCK2R^{-/-}$ mice relative to their wild-type littermates, and in the mesolimbic area a similar trend was seen. In the heterozygous $CCK2R^{+/-}$ mice, the density of D_2 receptors was somewhat elevated in the striatum, but not at all in the mesolimbic area. No differences in the affinity to [^{3}H] spiperone were observed (Paper I). Li and colleagues have demonstrated that CCK 8S induced the inhibition of D2 receptor affinity is larger in slice compared with the membrane preparation, suggesting that intact intracellular mechanisms and more intact membrane structure are required for an optimal CCK/D_2 receptor-receptor interaction (Li et al. 1995). Moreover, the results of the gene expression studies were in line with the stimulated dopaminergic projections from the mesencephalon to the mesolimbic structures of the CCK_2 receptor deficient mice (Paper III). Indeed, *Drd2* was down-regulated in the mesencephalon of the drug naïve homozygous $CCK2R^{-/-}$ mice. D_2 receptors in the mesencephalon are DA autoreceptors. These receptors regulate the synthesis and release of DA and it has been suggested that auto-receptor sub-sensitivity contributes to the enhanced release of DA (Pierce and Kalivas 1997). On the other hand, the expression of tyrosine hydroxylase gene, responsible for the synthesis of DA, was elevated in the mesolimbic structures of the drug naive genetically modified mice. The increased response to

apomorphine-induced motor suppression is probably related to the increased sensitivity of the striatal presynaptic DA receptors and a higher response to amphetamine in the homozygous CCK2R$^{-/-}$ animals is most likely related to the higher sensitivity of the striatal postsynaptic DA receptors, as the radioligand binding studies confirm. Moreover, Dauge and colleagues showed as well that the tone of dopaminergic system is increased in the forebrain structures of the male CCK_2 receptor deficient mice and the stimulating effect of amphetamine on the motor activity is stronger in these animals (Dauge et al. 2001).

To confirm the findings of acute experiments, indicated the altered function of DA system, we studied the effect of the repeated treatment with amphetamine in the CCK2R$^{-/-}$ mice (Papers III and IV). Caudate-putamen is involved in the voluntary movement as well as in the initiation, production and sequencing of motor behaviour in the development of addiction, whereas the NAc plays a major role in motivated and goal directed behaviours and in the development and expression of addiction (David et al. 2005). Taking into account this information it was feasible to study the addictive potency of the amphetamine CCK2R$^{-/-}$ mice. The effect of amphetamine was studied in conditioned place preference and motor sensitization experiments (Papers III and IV).

In the place conditioning test the effect of amphetamine was stronger in the wild-type CCK2R$^{+/+}$ mice compared to their homozygous CCK2R$^{-/-}$ littermates (Paper III). Only 1 mg/kg of amphetamine tended to induce a shift in the place-preference of the wild-type CCK2R$^{+/+}$ mice, whereas even 3 mg/kg of amphetamine did not induce a significant change in the preference of the homozygous CCK2R$^{-/-}$ mice. We know from earlier that infusion of CCK into the VTA potentiates amphetamine-induced conditioned place preference (Pettit and Mueller 1989). In accordance with our study, this effect could be attended to the activation of CCK2R. It has been demonstrated that the place conditioning induced by amphetamine is mediated via the NAc, and D_2 receptors there have a role in the induction of psychostimulant-induced place preference. The brain stimulation reward from the lateral hypothalamus is significantly attenuated in mice, lacking D_2 receptors (Elmer et al. 2005). In our study the repeated treatment with amphetamine caused a significant up-regulation of *Drd2* and *Tyhy* genes in the mesolimbic area of the wild-type CCK2R$^{+/+}$ mice, but not in their homozygous CCK2R$^{-/-}$ littermates (Paper III). The increased dopaminergic neurotransmission via D_2 receptors in the meso-limbic structures in the wild-type mice may be a reason for the differences in amphetamine-induced place preference established in two genotypes. The amphetamine-induced elevation of expression of *Tyhy* and *Drd1* genes in the striatum of the wild-type mice also may contribute to the stronger effect of DA agonist on the place conditioning in these animals. Dopaminergic mechanisms in the striatum have been shown to play an important role in habit formation and, therefore, in the development of addictive drug induced place preference (Gerdeman et al. 2003).

The evidence comes from previous studies that CCK modulates amphetamine induced motor sensitisation in rodents. The co-administration of CCK1R antagonist devazepide with amphetamine prevents the development of sensitization to this DA agonist. The simultaneous administration of CCK2R antagonist L-365,260 with amphetamine potentiates amphetamine-induced hyper-locomotion (Alttoa and Harro 2004). We found that a four-day treatment with the sub-threshold dose of amphetamine (1 mg/kg) induced a stronger motor sensitization in the homozygous $CCK2R^{-/-}$ mice compared to their wild-type $CCK2R^{+/+}$ littermates (Paper III). The administration of amphetamine increased both the distance travelled and the number of corner entries in the CCK2R deficient mice. The latter measure can be taken as a sign of repeated stereotyped behaviour. In the wild-type $CCK2R^{+/+}$ mice, only the number of corner entries was increased significantly. However, this elevation was not as marked as in the case of genetically modified mice. It has been shown that the VTA and mesolimbic dopaminergic system plays a major role in the development of sensitisation to the motor stimulant effect of amphetamine (Ben-Shahar and Ettenberg 1994). However, we found that the repeated administration of amphetamine induced a similar increase in the expression of *Tyhy* and *Comt* in the mesencephalon of both genotypes (Paper III). This is in line with the finding that chronic administration of amphetamine increases the levels of tyrosine hydroxylase, the rate-limiting enzyme in dopamine biosynthesis, in the VTA (Nestler 1992). The increase in the expression of *Tyhy* and *Comt* probably reflects an increase in the synthesis and metabolism of DA in the mesencephalon under the influence of amphetamine. The reason for a higher motor activity of the $CCK2R^{-/-}$ mice in this motor sensitisation experiment could be due to the altered dopamine D_2 autoreceptors activity. In the wild-type $CCK2R^{+/+}$ mice 4-day treatment with amphetamine induced a significant increase in the expression of *Drd2* genes, but at the same time, the expression level of *Drd2* gene in the amphetamine-treated homozygous $CCK2R^{-/-}$ mice was significantly lower compared to the respective group of the wild-type animals. This finding indicates to the lower DA autoreceptors activity in the $CCK2R^{-/-}$ mesencephalon and could be a reason for the stronger amphetamine induced motor sensitization in the homozygous $CCK2R^{-/-}$ mice. Many studies describe a critical role of mesencephalic A10 dopamine neurons in the induction of behavioural sensitization to amphetamine (Kalivas and Weber 1988; Vezina 1993; Vezina and Stewart 1989).

In the behavioural sensitisation test with 2 mg/kg amphetamine, the first administration suppressed the motor activity in the $CCK2^{-/-}$ mice but not in the $CCK2^{+/+}$ mice (Paper IV). Four subsequent amphetamine treatments induced a significant motor sensitisation in both $CCK2^{-/-}$ and $CCK2^{+/+}$ mice. However, in the $CCK2^{-/-}$ mice receiving amphetamine, the motor activity measured on day 4 was less pronounced. Sensitisation to amphetamine was confirmed by injecting amphetamine 21 days after the last experiment. Both $CCK2^{-/-}$ and $CCK2^{+/+}$ animals that had received amphetamine treatment for 4 days displayed a significant motor sensitisation and similar to day 4, the amphetamine-induced

motor effect was weaker in the $CCK2^{-/-}$ mice (Figure 9.). An explanation for a significantly weaker effect of the repeated treatment with amphetamine (2 mg/kg) in the $CCK2^{-/-}$ mice compared to their $CCK2^{+/+}$ littermates might be the increased sensitivity of striatal pre-synaptic dopamine D2 receptors, but some other, like an endocannabinoid related, mechanism could be also considered.

6.2. Alterations in endocannabinoid mechanisms due to the lack of CCK2R

An important finding of the current study is that the co-administration of the CB1 antagonist rimonabant with amphetamine attenuated the development of dopamine agonist-induced motor sensitization in mice, supporting the role of eCBs in the mechanisms of amphetamine-induced sensitization (Paper IV). This effect was absent in the $CCK2R^{-/-}$ mice; thus, it could be hypothesized that the presence of CCK2R is necessary for the development of eCBs-mediated amphetamine-induced sensitization. The co-administration of amphetamine and rimonabant for 4 days significantly decreased the distance travelled compared to both $CCK2R^{+/+}$ and $CCK2R^{-/-}$ animals treated with vehicle and amphetamine (Figure 9). Although the change was not statistically significant, rimonabant alone tended to decrease locomotion; one can speculate that this effect may have a non-specific origin. However, when animals were challenged with amphetamine 21 days later, the $CCK2R^{+/+}$ animals previously administered rimonabant and amphetamine displayed a clearly attenuated response to the psychostimulant, demonstrating that sensitization had been inhibited by rimonabant. Interestingly, previous rimonabant treatment did not change the development of sensitization to amphetamine in the $CCK2R^{-/-}$ animals (Paper IV). Our study is in agreement with previous studies demonstrating that eCBs participate in psychostimulant-induced hyperactivity mechanisms. In these studies, the CB1 receptor-deficient animals and the animals treated with CB1 antagonist were shown to be less responsive to the acute stimulating effects of amphetamine (Tzavara et al. 2009). Moreover, the invalidation of CB1 receptors resulted in the loss of amphetamine (1 mg/kg)-induced locomotor sensitization (Thiemann et al. 2008). However, in the latter study, a higher dose of rimonabant (3 mg/kg) than that used in our study (1 mg/kg) augmented the development of amphetamine sensitization. These contradictory findings may be due to the fact that rimonabant is a mixed antagonist/partial agonist of CB1 receptors that has been shown to induce hyperlocomotion with an ED50 of 4.7 mg/kg (Compton et al. 1996).

The effect of rimonabant on the $CCK2R^{-/-}$ mice was somewhat different. On the fourth day, the CB1 receptor antagonist inhibited amphetamine-induced locomotion in the $CCK2R^{-/-}$ mice. However, the experiment performed 21 days after the last treatment with rimonabant revealed that an amphetamine challenge induced the same degree of hyperlocomotion in both groups of animals that

were previously administered rimonabant and amphetamine as well as animals that received vehicle and amphetamine. Thus, to block the development of sensitisation to amphetamine by rimonabant, intact CCK2R signalling is necessary. This finding is in agreement with our previous study demonstrating that CCK participates in the eCB-system activation involved in the development of stress-induced analgesia (Kurrikoff et al. 2008). However, Kurrikoff and colleagues (Kurrikoff et al. 2008) demonstrated that stress-induced analgesia was accompanied by the increased expression of CB1 receptor gene in the mesolimbic area of the wild-type mice. This effect was not noted in the $CCK2R^{-/-}$ mice. Therefore, it is possible that the genetic invalidation of the CCK2R alters eCB-mediated signalling in the mesolimbic structures.

6.3. Alterations in endopiate mechanisms due to the lack of CCK2R

Mutant male mice, lacking CCK2R and crossed back three times to the C57/BL6 background, displayed impaired motor ability (Paper I). The locomotor activity of the $CCK2R^{+/-}$ and $CCK2R^{-/-}$ mice tended to be lower than in their wild-type littermates after the administration of saline and, in one experiment, this difference reached a statistical significance. The motor impairment of the $CCK2R^{+/-}$ and $CCK2R^{-/-}$ male mice crossed back three times to the C57/BL6 background was revealed clearly in the rota-rod test (Kõks et al. 2001). It is possible that the targeted mutation of CCK2R induces the motor disturbances in mice predominantly having the genes of a 129 SV strain. The vast majority of gene knock-out experiments have used a diverse collection of embryonic stem cells derived from sub-strain 129, and the phenotypic analysis is often confined to F_2 hybrid mice derived by crossing the 129 cells derived chimeras with another inbred strain, typically C57BL/6 and the importance of this genetic background in the evaluation of complex behavioural traits in mice has also been convincingly proved. For example, the role of 129/SV or C57BL/6 genetic background of DA D_2 deficient mice in open-field activity and rotarod performance has been characterised (Kelly et al. 1998). Congenic C57BL/6 homozygous D_2 receptor deficient mice have higher locomotor activity and better performance in rotarod then congenic 129/SV homozygousD_2 receptor deficient mice. C57BL/6 mice show much longer latencies to fall than 129Sv mice in the rotarod test (Homanics et al. 1999). The further back-grossing of mice to the C57BL/6 background reversed this suppression of motor activity (Raud et al. 2003; Paper II and III).

Dauge et al. demonstrated that the basal locomotor activity of the $CCK2R^{-/-}$ male mice, previously not habituated to the test conditions, was increased compared to their wild-type $CCK2R^{+/+}$ littermates (Dauge et al. 2001). The observed increase of the spontaneous motor activity in the CCK2R deficient mice compared with the wild-type $CCK2R^{+/+}$ animals, was suppressed by the administration of the D_2 receptos selective antagonist sulpiride, or of naloxone,

a nonselective competitive antagonist of opioid receptors (Dauge et al. 2001; Pommier et al. 2002). It could indicate that the elevation of this behavioural parameter in mice lacking CCK2R, is due to an increased function of endopioid and with its downstream related dopaminergic system. Differently from the study of Daugé et al. and Pommier et al. (Dauge et al. 2001; Pommier et al. 2002) we were unable to find any significant differences in the horizontal component of the locomotor activity of the six times to the C57BL/6 background back crossed wild-type and the mutant CCK2R$^{-/-}$ male mice, not habituated to the motility boxes (Paper II). In our experiment only the frequency of rearing was higher in mice without CCK2R. This discrepancy could be due to different experimental condition. It has been demonstrated that differences exist in behavioural results even in seemingly identical testing situation in different laboratories (Crabbe et al. 1999; Wahlsten et al. 2003). Dauge et al (2001) and Pommier et al. (2002) monitored horizontal movement of animals in dim plastic cages (255 x 205 cm) under the light intensity of 5 lux and in our experiment the illumination level of the transparent test boxes (44,8 x 44,8 x 45 cm) was ~400 lux. Locomotor activity in mice is sensitive to the context and both intra test and extra-test cues can influence behaviour. It is demonstrated that adult male C57BL/6 mice were in the dim open field more active, they covered a greater distance, moved faster and made more stops than in experiments under the bright light (Clark et al. 2006).

In our study the higher motor activity of the CCK2R$^{-/-}$ male mice became evident after the habituation session (Paper II). The repeated exposure of mice to the motility boxes caused a significant reduction of the locomotor activity in the wild-type mice. The described behavioural change was significantly weaker in the mice lacking CCK2R. Central CCK- and endopioids are important factors in adaptation and are activated in a novel environment. For an illustration, CCK antagonises morphine-induced antinociception in a novel, but not in a familiar environment (Wiertelak et al. 1992). Also, morphine induces a significant anxiolytic-like action in rats, not habituated to the experimental conditions, whereas morphine was ineffective in habituated rats (Kõks et al. 1999; Kõks et al., 2000). Therefore, the lack of CCK2R, a subtype of CCK receptors preferentially distributed in the brain, apparently attenuates a normal adaptation to the novel environment. Differently from the study of Pommier and colleagues (Pommier et al. 2002) in our experimental design, only a higher dose of naloxone 10 mg/kg reduced the horizontal component of the locomotor activity in mice, and this effect was similar in the wild-type and mutant mice. Nevertheless, naloxone 1 mg/kg antagonised the increased frequency of rearing established in the homozygous CCK2R$^{-/-}$ mice showing that the elevation of this behavioural parameter is probably due to the increased function of the endopioid system in mice lacking CCK2R (Paper II). Moreover, we established that naloxone caused a different effect in the wild-type and homozygous CCK2R$^{-/-}$ mice adapted to the motility boxes. In these mice the high dose of naloxone (10 mg/kg) induced a significant inhibition of the locomotor activity in the CCK2R$^{-/-}$, but not in the CCK2R$^{+/+}$ mice. It has been shown that naloxone

at this high dose is lacking selectivity for the subtypes of opioid receptors (Tsuda et al. 1996). Nevertheless, it is likely that the impaired locomotor adaptation of the $CCK2R^{-/-}$ mice to the novel environment is due to the increased function of the endopioid system.

We also studied the effect of naloxone on the place aversion conditioning paradigm of the $CCK2R^{-/-}$ mice (Paper II). In the place aversion conditioning experiments naloxone 1 mg/kg tended to cause conditioned place aversion in the wild-type mice, whereas the dose of 10 mg/kg caused a significant place aversion. By contrast, the naloxone-induced place aversion was weaker in the $CCK2R^{-/-}$ mice, because this dose of naloxone only tended to shift the behaviour of mutant mice from the preferred to non-preferred side. Naloxone as an opioiod receptor competitive antagonist reduces the effect of endopioids in the central nervous system and the development of weaker place aversion in mutant mice may reflect an increased tone of the endopioid system in these mice. It was shown that naloxone 10 mg/kg failed to produce conditioned place aversion in mu-opioid receptor-deficient mice, whereas the effect of kappa-opioid agonist U50,488H remained unchanged (Skoubis et al. 2001). This finding obviously supports the involvement of mu-opioid receptors in the mediation of naloxone-induced place aversion. This behavioural phenomenon can be induced by the local administration of naloxone into the VTA and NAc, but not into the striatum and medial prefrontal cortex (Shippenberg and Bals-Kubik 1995). Some evidence suggests that naloxone-induced action is not linked only to the mesolimbic dopaminergic system, because the blockade of mu-opioid receptors in the dorsal periaquaductal gray matter caused the conditioned place aversion (Sante et al. 2000). The role of periaquaductal gray matter in the regulation of pain sensitivity is well-known (Mason 1999). In the $CCK2R^{-/-}$ mice a significant reduction of pain sensitivity is established (Veraksitš et al. 2003) and therefore, it is possible to speculate that the increased tone of the endopioid system in this brain region could be linked to the reduced effect of naloxone in the $CCK2R^{-/-}$ mice. Moreover, to some extent this study tends to support the finding of pharmacological experiments showing that the CCK2R antagonists attenuate naloxone-induced place aversion in rats (Valverde and Roques 1998).

The administration of morphine 5 mg/kg tended to increase the horizontal component of locomotor activity and significantly inhibited the frequency of rearing in the habituate $CCK2R^{+/+}$ mice group. The higher dose of morphine, 10 mg/kg, induced a significant hyper-locomotion in the wild-type animals. By contrast, in the $CCK2R^{-/-}$ mice this dose of morphine induced significantly weaker motor activation (Paper II). This finding contradicts with the study of Pommier and colleagues (Pommier et al. 2002) demonstrating that the administration of morphine (6mg/kg) and inhibition of enkephalin metabolism increases the locomotor activity in mice without CCK2R significantly more. The reason for these differences is unclear but could be attributed to a different research design and distinct genetic background of the mice used in these studies. Indeed, Pommier and colleagues (Pommier et al. 2002) found that the affinity and density of opioid receptors were not changed in the whole brain of

mice without CCK2R, however, we established a decreased affinity and increased density of the opioid binding sites in the striatum of $CCK2R^{-/-}$ mice (Paper II). We measured the density of opioid receptors by [^{3}H]-diprenorphine in four distinct forebrain structures (the cerebral cortex, striatum, mesolimbic area, and hippocampus), and at least in these structures no decline in the density of opioid receptors was observed. Indeed, the number of opioid receptors was elevated in the dorsal striatum of mutant mice, whereas in the other structures no differences in the density of opioid receptors were established if the $CCK2R^{-/-}$ and wild-type animals were compared. On the other hand, the affinity of opioid receptors was reduced in the striatum. The reduced affinity of opioid receptors in the striatum could support the initial idea of Pommier and colleagues (Pommier et al. 2002) that the levels of endopioid peptides are increased in certain brain regions and therefore the affinity of opioid receptors is decreased. By contrast from the striatum the affinity of opioid receptors was increased in the cerebral cortex of the mutant $CCK2R^{-/-}$ mice compared to the wild-type littermates. It is shown that repeated treatments, daily multiple injection of escalating doses of morphine for few days; induced tolerance to locomotor effect of morphine; reduction the specific binding of [^{3}H]-DAMGO in the VTA and [^{3}H]-SCH2339 in the striatum, and an increase in the specific binding of [^{3}H]-raclopride in the striatum, which were detected in the receptors autoradiography experiment (Le Marec et al. 2011). We established a higher density of the [^{3}H]-spiperone binding sites in the $CCK2R^{-/-}$ mice dorsal striatum. These findings seem to support our hypothesis that a significantly weaker effect of morphine in the $CCK2R^{-/-}$mice could be caused by chronically elevated levels of endoopioids in the CNS.

The behavioural response to drugs of abuse is implicated to the mesolimbic dopaminergic pathways. The rewarding properties of opiates have been related to the changes in the mesolimbic dopamine activity (Wise and Bozarth 1987). Morphine has been shown to activate A 9 and A10 cells (Gysling and Wang 1983) and as a result, it enhances dopamine release in the striatum (Di Chiara and Imperato 1988). GABA afferents in the VTA provides inhibitory feedback to A10 via spontaneous inhibitory postsynaptic potentials (Steffensen et al. 1998). Mu-opioid receptors localised on GABA-ergic neurons are activated by opiates causing a hyperpolarisation that reduces GABA release, thereby disinhibiting dopamine cell firing (Johnson and North 1992; Leite-Morris et al. 2002; Leite-Morris et al. 2004). Chronically elevated endogenous opioid peptide levels in the $CCK2R^{-/-}$ mice could have a direct connection to the altered function of the mesolimbic dopaminergic system of these mice. It is likely that these endopioids up-regulate dopaminergic activity by disinhibition of A10 neurons via inhibiting local GABAergic interneurons (Johnson and North 1992; Nugent et al. 2007) and the increased tone of the midbrain dopaminergic neurons could be a downstream effect of the elevated function of the endopioid system due to the targeted mutation of CCK2R. We did not find any differences in the content of DA and its metabolites and in the density of opioid receptors and D_2 in the mesolimbic area, but the gene expression studies

reflect the increased tone of dopaminergic projections from the mesencephalon to the mesolimbic structures of the CCK2R$^{-/-}$ mice. *Drd2* gene, dopamine auroreceptor gene, was down-regulated in the mesencephalon and on the other hand, the expression of *Tyhy* gene, responsible for the synthesis of DA, was elevated in the mesolimbic structures of the drug naive genetically modified mice (Paper III).

6.3. Concluding remarks and future directions

The results of the present study show that the targeted mutation of the CCK2R gene induces substantial alterations in the function of DA, endopioid, and eCBs systems. However, this is not surprising, because CCK is co-localised with dopamine and OP within the same neurones and brain regions (Hökfelt et al. 1980a; Hökfelt et al. 1980b; Gall et al. 1987). Despite some discrepancies, probaby due to different experimental conditions, the results of the present study are in accordance with the earlier experiments conducted by Daugé and colleagues (Dauge et al. 2001; Pommier et al. 2002). Nevertheless, it should always be kept in mind that two mouse lines 129Sv and C57/Bl6, differing markedly by their behavioural and neurochemical parameters, are used for the generation of genetically modified animals. The evidence suggests that the genetic background has significantly stronger impact on the gene expression (Schalkwyk et al. 2007) and, therefore, on the behaviour of mice than the invalidation of certain genes (Abramov et al. 2008). Therefore, the genetic background of genetically modified mice has to be considered if their biochemical and behavioural phenotype is analysed. We found significant changes in the motor coordination and locomotor activity of mice with the dominating 129Sv genotype at the beginning of our study. It is well documented that the performance of 129Sv mice in the motor coordination and locomotor activity test is significantly inhibited compared to C57/Bl6 mice (Homanics et al. 1999; Võikar et al. 2001). The back-crossings to the C57/Bl6 genetic background apparently attenuated disturbances in the motor performance of the mice lacking CCK2R. However, as a matter of fact, only a part, but not all, of the effects induced by the targeted mutation of CCK2R are dependent on the genes of the 129Sv mice.

The targeted mutation of CCK2R causes impaired adaptation to the novel environment. As mentioned above, the pharmacological, behavioural and molecular biology analysis revealed significant alterations in the activity of dopamine, encopioid, and eCBs systems in the CCK2R$^{-/-}$ mice. It is important to stress that the changes occurring in these neurotransmitter systems are not similar throughout the brain, because the established alterations are localized in discrete brain regions and therefore related to the distinct behaviours. The peculiarity of the behavioural outcome obtained from the CCK2R$^{-/-}$ mice is the combination of different integrated mechanisms. For example, the amphetamine induced sensitisation in the CCK2R$^{-/-}$ mice could be less sensitive to

rimonabant, because eCBs are produced in smaller amounts in the striatal neurons. The reason for that could be an increased activity of the signal transduction related to the endopioid and D_2 receptors, as well as, the missing intracellular effect of the CCK2R activation.

The opioid competitive antagonist naloxone diminished the increased locomotion, a sign of poor adaptation in the $CCK2R^{-/-}$ mice and a low dose of amphetamine reduced exploratory activity more in the mutant $CCK2R^{-/-}$ than in the wild-type $CCK2R^{+/+}$ mice. Therefore, one could speculate that these homozygous $CCK2R^{-/-}$ deficient mice have some behavioural similarities with the children having attention-deficit/hyperactivity disorder (ADHD). ADHD is a complex behavioural, emotional and cognitive disorder that is characterized by its core symptoms of impulsivity, hyperactivity, distractibility, inattentiveness and cognitive impairment. The neurobiological cause of ADHD probably lies, at least to major degree, within the dysregulation of the brain catecholaminergic systems in the prefrontal cortex and its connections to the striatal areas. It has been proposed that the inattentive subtype of ADHD may arise due to a dysfunction of dopamine functioning in the inhibitory control of the frontal cortex and the hyperactive/impulsive subtype due to impairment of functioning in subcortical structures (Heal et al. 2008). Taking into account the behavioural changes and alterations of the dopeminergic systems in the forebrain structures due to the invalidation of CCK2 receptors one could suggest these animals as a potential model for the preclincial study of ADHD. Indeed, further research has to be planned and performed in this direction.

Moreover, as far as the DA system is concerned, the behavioural and biochemical effects of distinct groups of antipsychotic drugs (including haloperidol, aripiprazole and olanzapine) have to be explored in these mice. It is interesting to stress that patients suffering from schizophrenia have an increased sensitivity to the DA receptors and an enhanced response to the psychotomimetic action of amphetamine (Gainetdinov et al. 2001)**.** We have established something similar in mice lacking CCK2R. Taking into account the distinct changes in the effectiveness of morphine in the models of motor activity and place preference, it is of a great interest to explore the other motivational and addictive responses in the mice without CCK2R. In conclusion, we believe that this model can be of great importance for the pre-clinical screening of new drugs interacting with the neurotransmitter systems mediating motivational responses and psychotic behaviours.

7. CONCLUSIONS

1. Genetic deletion of CCK2R in mice increases the activity of the midbrain dopaminergic neurons. Although the concentration of DA and its metabolites in the brain tissue was not changed, the gene expression studies were in line with the pharmacological and behavioural experiments indicating enhanced activity of dopaminergic projections.
2. Pharmacological testing demonstrated that lack of CCK2R leads to the functional augmentation of the endopioid system and, therefore, changes the adaptation of mice in a novel environment.
3. The CCK and eCBs play a role in the development of amphetamine induced sensitization and intact CCK2R are necessary for the mediation of eCBs effect on the development amphetamine-induced sensitisation.

Genetic invalidation of CCK2R demonstrates that CCK plays a distinct role in the regulation of behaviours in the brain, it includes the regulation of motor, emotional and motivational mechanisms. Therefore, this animal model has potential to be applied for the pre-clinical screening of drugs targeting psycho-pharmacology.

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SUMMARY IN ESTONIAN

Dopamiini, endopioidi ja endokannabinoidi süsteemide funktsionaalsed muutused CCK2 retseptori puudulikkusega hiirtel

Koletsüstokiniin (CCK) on imetajate kesknärvisüsteemis laialdaselt levinud peptiidne neuromediaator, mis kaasmediaatorina osaleb erinevate kesknärvisüsteemi funktsioonide (hirm, valu, küllastustunne, motivatsioonide kujunemine, mälu, õppimine) regulatsioonis. Käesoleva töö eesmärk oli uurida CCKergilise süsteemi koostoimet dopamiinergilise, opioidergilise ja endokannabinoidergilise süsteemiga, rakendades selleks transgeense tehnoloogia võimalusi, hiirte käitumise uurimist, farmakoloogilisi ja molekulaarbioloogilisi teste. Täpsemateks eesmärkideks oli selgitada CCK2 retseptori geneetilise puudulikkusega ($CCK2R^{-/-}$) isastel hiirtel: 1) CCK ergiliste mehhanismide mõju aju dopamiinergiliste neuronite funktsioonile; 2) kuidas CCK2 retseptorite (CCK2R) geneetiline väljalülitamine muudab endopioidide süsteemi aktiivsust ja hiirte adaptatiivset käitumist; 3) endokannabinoidide rolli amfetamiini poolt indutseeritud sensibilisatsiooni kujunemises ning CCK2R võimalik seos sellega.

Selgus, et CCK2R geneetiline väljalülitamine põhjustab funktsionaalseid muutusi hiirte mesolimbilises dopamiinergilises süsteemis. $CCK2R^{-/-}$ hiirte suurenenud tundlikus mitteselektiivse dopamiini D_1 ja D_2 retseptorite agonisti apomorfiini motoorikat pärssiva toime suhtes, viitab presünaptiliste D_2 retseptorite funktsiooni suurenemisele. Sünapsidest dopamiini vabastava amfetamiini annuse 3 mg/kg motoorikat stimuleeriv toime oli $CCK2R^{-/-}$ hiirtel nõrgem ja annuse 6 mg/kg toime tugevam kui nende CCK2 retseptoriga ($CCK2R^{+/+}$) pesakaaslastel. See viitab postsünaptiliste dopamiinirtetseptorite tundlikkuse muutusele. Radioligandi sidumiskatse tulemused näitasidki $CCK2R^{-/-}$ hiirtel dorsaalses juttkehas suuremat D_2 retseptorite tihedust. Kuigi dopamiini ja selle metaboliitide taseme määramisel erinevates ajustruktuurides muutusi ei ilmnenud, siis lisaks farmakoloogilistele käitumiskatsetele toetasid ka geenieksprессiooni analüüsi tulemused hüpoteesi, et $CCK2R^{-/-}$ hiirtel on mesolimbilistes struktuurides tõusnud dopamiinergiliste neuronite tooniline aktiivsus. Näiteks leidsime keskajus madalama dopamiini D_2 retseptorite mRNA taseme ja ventraalses juttkehas tõusnud türosiinihüdroksülaasi mRNA taseme. Keskaju D_2 retseptorid on põhilises osas dopamiinergilistele neuronitele negatiivset tagasisidekontrolli pakkuvad autoretseptorid ja türosiinihüdroksülaas on ensüüm, mis määrab dopamiini sünteesi aktiivsuse.

Mesolimbiline dopamiinergiline süsteem täidab keskset rolli sõltuvushäirete tekkes ja seetõttu uurisime amfetamiini sõltuvust tekitavat toimet $CCK2R^{-/-}$ hiirtel. Selgus, et amfetamiini kohaeelistust tingiv toime nendel hiirel oli vähenenud. Motoorse sensibilisatsiooni testis omasid erinevad annused erinevat toimet. $CCK2R^{-/-}$ hiirte sensibilisatsioon amfetamiini annusega 1 mg/kg põhjustas suurema motoorse aktivatsiooni ja annus 2 mg/kg väiksema motoorse aktivatsiooni kui nende $CCK2R^{+/+}$ pesakaaslastel. Geenieksprессiooni analüüs

näitas, et sensibiliseerimine amfetaminiga 1 mg/kg põhjustas $CCK2R^{+/+}$ hiirtel dorsaalses juttkehas D_1 retseptorite ja ventraalses juttkehas D_2 retseptorite mRNA taseme olulise tõusu, $CCK2R^{-/-}$ hiirtel aga mitte. Samas tuleb arvestada, et lisaks muutunud dopamiini retseptorite tundlikkusele võivad mõjutused tulla ka mujalt, mittedopamiinergilistest mehhanismidest.

Uurisime näiteks endokannabinoidide võimalikku rolli amfetamiinist tingitud motoorse sensibilisatsiooni tekkes, kasutades selleks rimonabanti, endokannabinoidide CB1 retseptori konkureerivat antagonisti. Eelnev rimonabandi manustamine pärssis $CCK2R^{+/+}$ hiirtel sensibilisatsiooni väljakujunemist, $CCK2R^{-/-}$ hiirtel aga mitte. Kakskümmend üks päeva peale sensibiliseerimist manustatud ühekordne amfetamiini annus (2 mg/kg) põhjustas sensibiliseerimise perioodis amfetamiinile eelnevalt rimonabanti saanud $CCK2R^{+/+}$ hiirtel oluliselt väiksema motoorse aktivatsiooni kui sama genotüübiga vaid amfetamiiniga sensibiliseeritud hiirtel. $CCK2R^{-/-}$ hiirel põhjustas aga kakskümmend üks päeva peale sensibiliseerimist manustatud ühekordne amfetamiini annus (2 mg/kg) nii sensibiliseerimise perioodis amfetamiinile eelnevalt rimonabanti saanud hiirel, kui ka vaid amfetamiini saanud hiirel ühesuguse motoorse aktivatsiooni. See viitab endokannabinoidide osalusele amfetamiinist tingitud sensibilisatsiooni mehhanismides ning, et nende mehhanismide käivitumine eeldab toimivat CCK2R poolt vahendatud signaaliülekannet. Mesolimbiliste dopamiinergiliste neuronite toonilist aktiivsust võib suurendada ka tõusnud endopioidide süsteemi aktiivsus. $CCK2R^{-/-}$ hiirel esines väiksem keskkonnaga adaptatsiooni võime. Uudse keskkonna korduval eksponeerimisel hiirtele toimus selles keskkonnas olevate $CCK2R^{+/+}$ hiirte motoorse aktiivsuse vähenemine, $CCK2R^{-/-}$ hiirtel jääb aga motoorne aktiivsus oluliselt kõrgemaks. Motoorse aktiivsuse erinevuse kõrvaldas μ-opioidretseptori konkureeriva antagonisti naloksooni manustamine. See viitab võimalusele, et suurem motoorne aktiivsus on tingitud kõrgemast endopioidide aktiivsusest $CCK2R^{-/-}$ hiirte kesknärvisüsteemis. Endopioidi-süsteemi aktiivsuse muutusele viitab ka erinev motoorne reaktsioon morfiini erinevatele annustele ning radioligandi sidumiskatsega leitud suurem opioidiretseptorite afiinsus ajukoores ning suurem tihedus ja väiksem afiinsus $CCK2R^{-/-}$ hiirte dorsaalses juttkehas. Opiaatide motoorset aktiivsust suurendav toime põhineb mesolimbiliste dopamiinergiliste neuronite aktiivsuse tõstmisel. Opiaadid pidurdavad tooniliselt aktiivseid GABA-ergilisi vaheneuroneid, mis kontrollivad vaheajus dopamiinergiliste neuronite aktiivsust. See tähendab, et CCK2R geneetiline väljalülitamine tingib muutusi nii dopamiinergilise, enendokannabinoidergilise ja ka opioidergilise süsteemi talitluses. Muutuste põhjused võivad olla otsesed, CCK2R poolt vahendatud mediaatorite sünteesi ja vabanemist reguleeriva signaali puudumine, või hoopis kaudsed, tingitud erinevate mediaatorsüsteemide muutunud koostoimest. Töö tulemused kinnitavad, et CCK omab kindlat rolli hiirte käitumise motoorsetes, emotsionaalsetes ja motivatsioonilistes mehhanismides ja, et $CCK2R^{-/-}$ hiired võivad osutuda potentsiaalseteks mudeliteks erinevate psühhofarmakonide prekliinilisel testimisel.

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Rünkorg, K.; Orav, L.; Kõks, S.; Matsui, T.; Volke, V.; Vasar, E. (2012). Rimonabant attenuates amphetamine sensitisation in a CCK2 receptor-dependent manner. Behavioural Brain Research, 226(1), 335–339

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